REMARKS

Applicants would first like to thank Examiner Anderson for the Interview of April 9, 2009. Upon entry of the present amendment, claims 44, 47, 49, 52, 54, 55 and 57-67 are pending and under examination. Claims 5-7, 10, 12, and 35-39 remain withdrawn from further consideration in view of the Restriction Requirement mailed 12/20/06, and Applicants' elections filed 1/19/07. Claims 1-4, 8, 9, 11, 13-34, 40-43, 45, 46, 48, 50, 51, 53, 56, 68 and 69 have been cancelled. Claims 44, 47, 49, 52, 54, 55, 57, 59, 60 and 64-67 have been amended.

Specifically, claims 44, 47, 49, and 52, have been amended to indicate that the pharmaceutical composition is administered before, during, or after at least one chemotherapeutic agent. Support for this amendment may be found at the very least at page 16, lines 20-21. Claim 67 has been amended to clarify that the method of claim 44 further comprises surgical resection, radiation therapy, chemotherapy, hormone therapy, anti-tumor vaccination, antibody based therapy, cytokine based therapy, whole body irradiation, bone marrow transplantation, and peripheral stem cell transplantation. The remaining claims have been amended accordingly to correct dependency. No prohibited new matter has been added with the forgoing amendments.

Rejections under 35 U.S.C. § 112, 1st paragraph

Claims 1, 4, 13, 30-34, 44-45, 49-50 and 54-68 are rejected by the Examiner for failing to comply with the written description requirement of 35 U.S.C. § 112, first paragraph. Specifically, the Examiner has rejected the claims for lacking support for administering compositions consisting essentially of 1-methyl-D-tryptophan. Although the Examiner

acknowledges that the "instant specification discloses methods of treating cancer and tumors comprising administering an inhibitor of indoleamine-2,3-dioxygenase and administering at least one chemotherapeutic agent" (emphasis in Office Action), the Examiner asserts there is no implicit or inherent support for compositions *consisting essentially of* 1-methyl-D-tryptophan Applicants respectfully disagree.

The term "consisting essentially of" is a well known and widely used transitional phrase. According to the M.P.E.P., "[t]he transitional phrase 'consisting essentially of' limits the scope of a claim to the specified materials or steps 'and those that do not materially affect the basic and novel characteristic(s)' of the claimed invention." M.P.E.P. § 2211.03, *citing* In re Herz, 537 F.2d 549, 551-52 (C.C.P.A. 1796). Therefore, a composition "consisting essentially of 1-methyl-D-tryptophan" is a composition which contains 1-methyl-D-tryptophan, and may also contain other materials which "do not materially affect" the composition, such as inactive ingredients/excipients (i.e. diluents, buffers, binders, disintegrants, surface active agents, thickeners, lubricants or preservatives, as claimed in claim 65). Furthermore, there are several compositions "consisting essentially of 1-methyl-D-tryptophan" disclosed in the specification. For example, parenteral formulations are described at page 19, lines 1-11 and enteral formulations are described at page 19, lines 12-18.

Given the phrase "consisting essentially of" is commonly used and accepted patent claim terminology, and given that the specification clearly discloses examples of formulations consisting essentially of 1-methyl-D-tryptophan and various excipients, buffers, etc., Applicants presume the Examiner objects to the phrase "consisting essentially of" in light of the disclosure that inhibitors of the invention are used in combination with at least one additional agent to achieve a synergistic effect. Applicants respectfully note, however, that the application discloses

that the administration of the inhibitor may take place before, during or after administration of the chemotherapeutic agent, as acknowledged by the Examiner on page 4 of the Office Action (see page 16, lines 20-21 of the specification). Administration before or after the additional agent implies that the claimed inhibitors may be administered in a composition consisting essentially of only the inhibitors.

Nevertheless, in an effort to expedite prosecution, Applicants have cancelled claims 1, 4, 13, 30-34, 45 50, 56 and 68. In addition, Applicants have amended claims 44 and 49 to indicate that the composition consisting essentially of 1-methyl-D-tryptophan is administered, before, during or after administration of at least one chemotherapeutic agent. Reconsideration and withdrawal of the written description rejection are respectfully requested.

Claims 1-2, 4, 13, 30-34 and 44-69 were rejected under 35 U.S.C. § 112, first paragraph, because, according to the Examiner, while the specification is enabling for treating cancer, augmenting the rejection of tumor cells, or reducing or slowing tumor growth with 1-methyl-D-tryptophan and cyclophosphamide, it does not reasonably provide enablement for treating cancer, augmenting rejection of tumor cells, or reducing or slowing tumor growth with 1-methyl-D-tryptophan and other chemotherapeutic agents, as claimed in claims 1-2, 4, 13, 30-34, 45, 48, 50, 53-54 and 68-69; or treating cancer or reducing, augmenting rejection of tumor cells, or reducing or slowing tumor growth with 1-methyl-D-tryptophan alone, as claimed in claims 44, 46-47, 49, 51-52 and 55-67.

Without agreeing with the rejection, Applicants have cancelled claims 1-2, 4, 30-34, 45, 46, 48, 50, 51, 53, 56, 68 and 69. Additionally, Applicants have amended claims directed to methods of treating cancer, augmenting rejection of tumor cells, or reducing or slowing tumor growth with 1-methyl-D-tryptophan to also entail the administration of at least one

chemotherapeutic agent, before, during or after administration of 1-methyl-D-tryptophan. Accordingly, that aspect of the rejection is moot. With regard to the scope of enablement rejection regarding other chemotherapeutic agents, Applicants respectfully submit that the claimed invention is readily applicable to use with any chemotherapeutic agent as evidenced by the attached publication by the inventors (Hou, *et al.*, (2007) Inhibition of Indoleamine 2,3-Dioxygenase in Dendritic Cells by Stereoisomers of 1-Methyl-Tryptophan Correlates with Antitumor Responses, Cancer Res. 67:792-801) ("Hou")(Exhibit A).

Specifically, Hou reports that 1-methyl-D-tryptophan is effective in treating several types of cancers with 1-methyl-D-tryptophan in combination with several different kinds of chemotherapeutic agents, including cyclophosphamide, paclitaxel and gemcitabine. The paper clearly shows that 1-methyl-D-tryptophan given in combination with cyclophosphamide (Figure 5A) or gemcytabine (Figure 5B) reduces the size of tumors *in vivo* in a melanoma model to a significantly greater extent than either chemotherapeutic or 1-methyl-D-tryptophan alone (see discussion at page 797, column 1). The paper also reports significantly improved results with the D isomer and paclitaxel as compared to the L isomer and paclitaxel for treatment of tumors in a breast cancer model (See Figure 6 and page 797, column 2). Furthermore, paclitaxel given in combination with 1-methyl-D-tryptophan was significantly more effective than paclitaxel given alone in reducing tumor size in mice (Figure 6B). Applicants also note, according to this report, that in at least one model, 1-methyl-D-tryptophan was effective alone, without an additional chemotherapeutic agent, in reducing tumor size. (Figure 5C). Thus, taken together, the evidence in the Hou paper shows that 1-methyl-D-tryptophan is effective in treating solid tumor cancers when combined with a number of known chemotherapeutic agents according to the methods disclosed in the specification. Moreover, the attached exhibits B-E indicate that treatment with 1-

dimethyl-D-tryptophan is also applicable to leukemia. *See* Curti, *et al.* (2007) Modulation of Tryptophan Catabolism by Human Leukemic Cells Results in the Conversion of $CD25^-$ into $CD25^+$ T Regulatory Cells, Blood 109:2871-2877 (Exhibit B); Curti, *et al.* (2007) Acute Myeloid Leukemia Cells Constitutively Express the Immunoregulatory Enzyme Indoleamine 2,3-Dioxygenase, Leukemia 21:353-355 (Exhibit C); Corm, *et al.* (2009) Indoleamine 2,3-Dioxygenase Activity of Acute Myeloid Leukemia Cells can be Measured from Patients' Sera by HPLC and is Inducible by IFN-γ, Leukemia Res. 33:490-494 (Exhibit D); and Chamuleau, *et al.* (2008) High *INDO* (Indoleamine 2,3-Dioxygenase) mRNA Level in Blasts of Acute Myeloid Leukemic Patients Predicts Poor Clinical Outcome, Haematologica 93:1894-1898 (Exhibit E). Accordingly, the scope of the amended claims is fully enabled. Reconsideration and withdrawal of the enablement rejection is earnestly solicited.

Double Patenting

The Examiner has provisionally rejected claims 1, 2, 4, 13, 30-34 and 44-69 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 2, 6-7, 10, 17-18, and 97-132 of copending Application No. 10/780,150. Claims 1, 2, 4, 13, 30-34, 45, 46, 48, 50, 51, 53, 56, 68 and 69 have been cancelled by the present amendment. Applicants respectfully request that this rejection as it applies to the remaining claims be held in abeyance until indication of otherwise allowable subject matter, and Applicants will consider at that time whether the submission of a terminal disclaimer would be appropriate.

CONCLUSION

In view of the foregoing, Applicants respectfully submit that no further impediments exist to the allowance of this application and, therefore, requests an indication of allowability. However, the Examiner is urged to call the undersigned if any questions or comments arise, or if any further issues remain to be resolved in order to expedite this application to allowance.

The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 50-1283.

Dated: April 14, 2009

Respectfully submitted,
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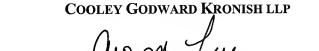
By: 
Alyson C. Fuller

Reg. No. 61,844

Exhibit A

Inhibition of Indoleamine 2,3-Dioxygenase in Dendritic Cells by Stereoisomers of 1-Methyl-Tryptophan Correlates with Antitumor Responses

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[1]Immunotherapy Center and Departments of [2]Pediatrics, [3]Medicine, and [4]Biostatistics, Medical College of Georgia, Augusta, Georgia; [5]Lankenau Institute for Medical Research, Wynnewood, Pennsylvania; [6]NewLink Genetics Corporation, Ames, Iowa; and [7]Department of Pathology, Anatomy, and Cell Biology, Jefferson Medical College, Philadelphia, Pennsylvania

Abstract

Indoleamine 2,3-dioxygenase (IDO) is an immunosuppressive enzyme that contributes to tolerance in a number of biological settings. In cancer, IDO activity may help promote acquired tolerance to tumor antigens. The IDO inhibitor 1-methyl-tryptophan is being developed for clinical trials. However, 1-methyl-tryptophan exists in two stereoisomers with potentially different biological properties, and it has been unclear which isomer might be preferable for initial development. In this study, we provide evidence that the D and L stereoisomers exhibit important cell type–specific variations in activity. The L isomer was the more potent inhibitor of IDO activity using the purified enzyme and in HeLa cell–based assays. However, the D isomer was significantly more effective in reversing the suppression of T cells created by IDO-expressing dendritic cells, using both human monocyte–derived dendritic cells and murine dendritic cells isolated directly from tumor-draining lymph nodes. *In vivo*, the D isomer was more efficacious as an anticancer agent in chemo-immunotherapy regimens using cyclophosphamide, paclitaxel, or gemcitabine, when tested in mouse models of transplantable melanoma and transplantable and autochthonous breast cancer. The D isomer of 1-methyl-tryptophan specifically targeted the *IDO* gene because the antitumor effect of D-1-methyl-tryptophan was completely lost in mice with a disruption of the *IDO* gene (IDO-knockout mice). Taken together, our findings support the suitability of D-1-methyl-tryptophan for human trials aiming to assess the utility of IDO inhibition to block host-mediated immunosuppression and enhance antitumor immunity in the setting of combined chemo-immunotherapy regimens. [Cancer Res 2007;67(2):792–801]

Introduction

The immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) has been implicated as an immunosuppressive and tolerogenic mechanism contributing to maternal tolerance toward the allogeneic fetus (1), regulation of autoimmune disorders (2–5), and suppression of transplant rejection (6, 7). IDO can also be expressed by cancer cells in a variety of human malignancies (8, 9). In murine models, transfection of immunogenic tumor cell lines with recombinant IDO renders them immunosuppressive and lethally progressive *in vivo*, even in the face of otherwise protective T-cell immunity (8). In humans, expression of IDO by ovarian and colorectal cancer cells has been found to be a significant predictor of poor prognosis (9, 10).

IDO can also be expressed by host antigen-presenting cells (APC). APCs with the potential to express IDO include human monocyte–derived macrophages (11), human monocyte–derived dendritic cells cultured under specific conditions (12–19), and certain subsets of murine dendritic cells (20–25). In murine tumor models, IDO$^+$ dendritic cells displaying a plasmacytoid phenotype (CD11c$^+$B220$^+$) have been found at increased levels in tumor-draining lymph nodes (22). These have been shown to suppress T-cell responses *in vitro* and create antigen-specific T-cell anergy *in vivo* (22, 25). In humans, IDO$^+$ cells of host origin have been shown in draining lymph nodes of patients with melanoma, breast cancer, and other tumors (13, 22, 26, 27). In patients with malignant melanoma, the presence of these IDO-expressing cells in sentinel lymph node biopsies was correlated with significantly worse clinical outcome (22, 28). Thus, expression of IDO, either by host cells or by tumor cells, seems associated with poor outcome in a number of clinical settings.

These findings have prompted interest in development of IDO inhibitor drugs for cancer immunotherapy (29). The most widely studied of these has been 1-methyl-tryptophan (30–32). Recently, it was shown that 1-methyl-tryptophan displays marked synergy with a number of clinically relevant chemotherapeutic agents when used in combined chemo-immunotherapy regimens (33). In that study, the combination of 1-methyl-tryptophan with cyclophosphamide, cisplatin, doxorubicin, or paclitaxel was able to cause regression of established tumors in a demanding model of autochthonous HER-2/*neu*–induced murine breast cancers (33). From a clinical standpoint, combining an immunomodulatory agent, such as 1-methyl-tryptophan, with conventional chemotherapy drugs represents an attractive strategy, and a sound mechanistic rationale supporting such chemo-immunotherapy approaches is now being elucidated (34–36).

However, a key unanswered question regarding 1-methyl-tryptophan has been which of the two available stereoisomers (D and L) should be developed initially for clinical trials. The two isomers differ significantly in their effects on the recombinant IDO enzyme *in vitro* (37), and they could potentially have different biological effects, bioavailability, and off-target toxicities. Most of the studies in the literature have employed the racemic (DL) mixture of 1-methyl-tryptophan comprising both isomers, thus leaving unanswered the question of which stereoisomer would be

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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best suited for use in chemo-immunotherapy regimens. The goal of the present study was to compare the biological activity of the D and L isomers of 1-methyl-tryptophan *in vitro* and *in vivo*, to ask whether their pattern of efficacy *in vitro* correlated with their observed antitumor effect *in vivo*.

Materials and Methods

Additional methods available online. Detailed description of mice, published methods, and statistical analyses are available online at http://cancerres.aacrjournals.org/.

Reagents. 1-Methyl-D-tryptophan (45,248-3), 1-methyl-L-tryptophan (44,743-9), and 1-methyl-DL-tryptophan (86,064-6) were obtained from Sigma-Aldrich (St. Louis, MO). For *in vitro* use, these were prepared as a 20 mmol/L stock in 0.1 N NaOH, adjusted to pH 7.4, and stored at −20°C protected from light.

Autochthonous breast cancer model. Multiparous female MMTV-*Neu* mice, maintained as described (33), have a high incidence of autochthonous mammary gland carcinomas. Tumor-bearing mice were enrolled randomly into experimental groups when tumors reached 0.5 to 1.0 cm in diameter. Tumor volume was measured at the beginning and end of the 2-week treatment period.

B16F10 and 4T1 tumor models. B16F10 melanoma (American Type Culture Collection, Manassas, VA) were established in B6 mice by s.c. injection of 5×10^4 cultured cells. B78H1-GM-CSF (38), gift of H. Levitsky, (Johns Hopkins University, Baltimore, MD) was implanted by s.c. injection of 1×10^6 cells. Orthogonal diameters were measured, and the *x*·*y* product (tumor area) was reported. The use of the orthotopically implanted 4T1 breast cancer line (39) has been described in detail (40). Tumors were implanted by injection of 1×10^4 cells in 50 μL volume into the mammary fat pad of 6- to 10-week-old BALB/c females. In some experiments, luciferase-transfected 4T1 cells (4T1-luc) were used for bioluminescence imaging, as described in the Supplementary Material.

Administration of 1-methyl-tryptophan and chemotherapeutic agents. Detailed protocols for administration of 1-methyl-tryptophan, orally and by s.c. pellets, in conjunction with chemotherapy, are given in the Supplementary Material.

Human and mouse mixed lymphocyte reactions. Human and murine allogeneic mixed lymphocyte reactions (allo-MLR) were done as detailed in the Supplementary Material and have been previously described (14, 22).

Western blots. Western blots were done using affinity-purified polyclonal rabbit antibody against peptides from the NH_2-terminal and COOH-terminal portion of human IDO, as previously described (13) and as specified in detail in the Supplementary Material.

Results

Cooperativity effect of s.c. DL-1-methyl-tryptophan with chemotherapy or radiation in B16F10 melanoma. We first evaluated the racemic DL mixture of 1-methyl-tryptophan as a component of chemo-immunotherapy using three tumor models: a stringent established (day 7) B16F10 melanoma, orthotopically implanted 4T1 breast carcinoma, and autochthonous breast tumors arising in HER-2/*neu*–transgenic mice. Figure 1*A* shows established B16F10 tumors treated with DL-1-methyl-tryptophan (20 mg/d by 14-day s.c. copolymer pellet; ref. 1), with or without a single injection of cyclophosphamide (150 mg/kg). DL-1-methyl-tryptophan alone had no effect on tumor growth, and cyclophosphamide alone induced only a transient growth delay. However, the combination of DL-1-methyl-tryptophan + cyclophosphamide resulted in a sustained growth delay and prolonged survival. In all experiments, the end of the study period was defined as the time when all of the mice in the vehicle-only group reached their ethical surrogate end point (tumor area ≥300 mm^2). At the point when all mice in the control group had reached this end point, all mice in the

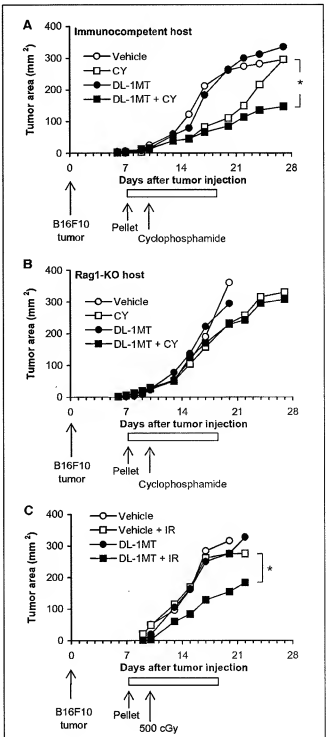
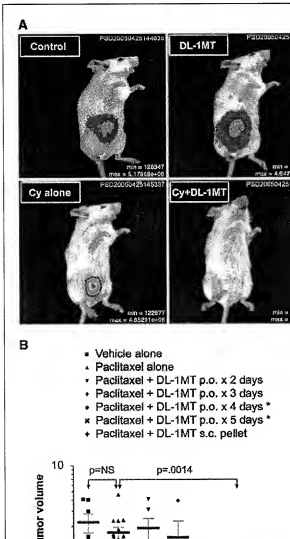


Figure 1. Effect of parenteral DL-1-methyl-tryptophan (*DL-1MT*) in B16F10 tumors. *A*, B16F10 tumors were implanted in syngeneic C57BL/6 mice. Beginning on day 7, mice were treated as shown with timed release s.c. pellets of DL-1-methyl-tryptophan (20 mg/d) plus cyclophosphamide (*CY*; 150 mg/kg i.p. × 1 dose). Three identical experiments were done (a representative example is shown), and the pooled results were analyzed in a three-experiment × 2 group ANOVA. *, $P < 0.05$. *B*, identical experimental design showing that the effect of DL-1-methyl-tryptophan was lost when hosts were immunodeficient Rag1-KO. Groups were not significantly different by ANOVA. *C*, similar experimental design, except that 500 cGy of whole-body cesium-137 irradiation replaced the cyclophosphamide. One of four similar experiments. *, $P < 0.05$, ANOVA.

DL-1-methyl-tryptophan + cyclophosphamide group were still surviving. Figure 1*B* shows that the effect of DL-1-methyl-tryptophan was lost in immunodeficient Rag1-knockout (Rag1-KO) hosts, indicating that the antitumor effect of DL-1-methyl-tryptophan was entirely immune mediated.

Whole-body irradiation has many of the same effects as chemotherapy when combined with antitumor immunotherapy (41). We tested DL-1-methyl-tryptophan in combination with 500 cGy whole-body irradiation (Fig. 1*C*). In these experiments, there was considerable variability in the effect of the radiation component alone on tumor growth, but in all experiments, the effect of DL-1-methyl-tryptophan plus radiation was superior to radiation alone.



Cooperativity between oral DL-1-methyl-tryptophan and cyclophosphamide in treating 4T1 breast carcinoma isografts. We next asked whether DL-1-methyl-tryptophan showed efficacy via the oral route. For these studies, we tested chemo-immunotherapy of the poorly immunogenic 4T1 breast tumor model, implanted orthotopically in mammary tissue of syngeneic hosts. Because orthotopic 4T1 tumors are highly invasive and their margins are difficult to measure conventionally, we followed the tumor size using luciferase-transfected 4T1 (4T1-luc) tumors imaged following luciferin challenge. Oral DL-1-methyl-tryptophan was given by gavage twice daily, five times a week, combined with a weekly single i.p. dose of cyclophosphamide, beginning at the time of tumor implantation. As shown in representative scans in Fig. 2*A*, cyclophosphamide alone produced a modest reduction in tumor size, but the combination of cyclophosphamide + DL-1-methyl-tryptophan produced a marked decrease in tumor size (survival studies in this model are presented below).

Oral administration of DL-1-methyl-tryptophan in combination with paclitaxel can elicit regression of autochthonous breast tumors. We next tested the efficacy of varying durations of oral DL-1-methyl-tryptophan in combination with paclitaxel for the treatment of autochthonous tumors arising in MMTV-*Neu* mice (33). Mice with tumors were randomly assigned to treatment with paclitaxel for 2 weeks, with or without addition of 2 to 5 days of oral DL-1-methyl-tryptophan during the first week, as indicated in Fig. 2*B*. Paclitaxel alone caused a minor reduction in the rate of tumor growth, but tumors continued to increase in size during the study period despite paclitaxel. The addition of oral DL-1-methyl-tryptophan produced a progressive reduction in the rate of tumor growth with increasing duration of 1-methyl-tryptophan, such that treatment with 4 and 5 days of DL-1-methyl-tryptophan reversed tumor growth, and caused regression of the established tumors during the treatment period. Five days of administration via the oral route was at least as effective as parenteral delivery of the drug at a comparable daily dose, using implantable s.c. pellet (the last treatment group and the route reported in our previous study; ref. 33).

***In vitro* comparison of D versus L isomers of 1-methyl-tryptophan.** We next used *in vitro* models to compare the different isomers of 1-methyl-tryptophan for their biological effects, using two readouts: (*a*) activity of the IDO enzyme measured as

Figure 2. Oral DL-1-methyl-tryptophan in orthotopic 4T1 and autochthonous MMTV-*Neu* tumors. *A*, orthotopic tumor isografts were established in the mammary fat pad. Treatment was initiated concurrent with tumor challenge, using cyclophosphamide i.p. at 100 mg/kg, once a week and DL-1-methyl-tryptophan oral gavage at 400 mg/kg per dose, twice daily, five times a week. Bioluminescence imaging of 4T1 tumor cell lines transfected with luciferase, showing the effect of each treatment on tumor burden. Treatment received by each mouse is indicated. Images were produced at 4 wks following the initiation of treatment. *B*, MMTV-*Neu* mice bearing 0.5 to 1.0 cm spontaneous tumors were treated for 2 wks with either vehicle alone, paclitaxel alone (13.3 mg/kg i.v. q. M/W/F), or paclitaxel plus oral DL-1-methyl-tryptophan (400 mg/kg i.v. twice daily, given for up to 5 d during the first week, as indicated in the legend). Paclitaxel was given i.v. at over the 2-wk treatment period. The last group received s.c. pellets of 1-methyl-tryptophan, as in Fig. 1. Fold changes in individual tumor volumes over the 2-wk period are plotted for each group. *Points*, mean fold change for each group (also listed in the box below the graph); *bars*, SE. *, fully regressed tumors are included in the calculation of the mean and SE. For the statistical analyses (*arrows*), the two comparisons of interest were vehicle alone versus paclitaxel alone and paclitaxel alone versus paclitaxel + D-1-methyl-tryptophan × 5 d. Significance was determined at $P < 0.025$ using a two-group Wilcoxon exact test.

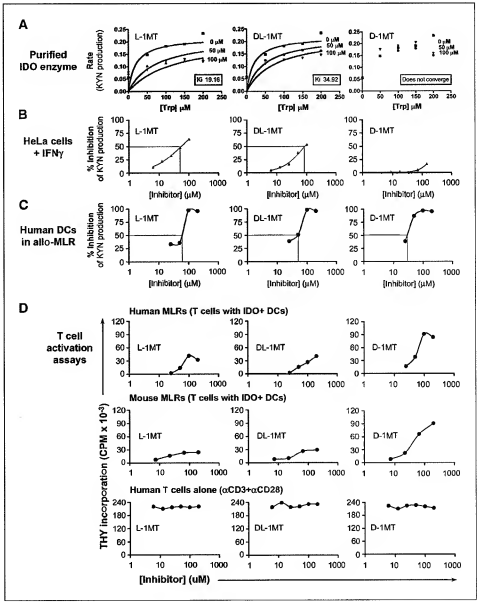


Figure 3. Effect of different isomers on *in vitro* enzyme assays and T-cell proliferation. *A*, enzyme kinetics, measured as kynurenine (*KYN*) production in cell-free assay, for purified recombinant human IDO, showing the effect of the L, DL, and D forms of 1-methyl-tryptophan in the presence of varying concentrations of L-tryptophan substrate. *B*, intracellular IDO enzyme activity (measured as kynurenine production in culture supernatants) by IFNγ-activated HeLa cells, showing inhibition by different isomers of 1-methyl-tryptophan. % Inhibition of maximal kynurenine production; lines show interpolated EC_{50} for each isomer. *C*, intracellular IDO activity (kynurenine production in MLR supernatants) by human monocyte–derived dendritic cells (*DC*) activated in allo-MLRs; lines show EC_{50}. Combined average of three experiments using three different donors. *D*, effect of 1-methyl-tryptophan isomers on T-cell proliferative responses. Proliferation was measured by thymidine incorporation in allo-MLRs using either human T cells stimulated by IDO-expressing human monocyte–derived dendritic cells (1 of 10 experiments, using a variety of different donor combinations), or mouse T cells stimulated by IDO-expressing plasmacytoid dendritic cells from tumor-draining lymph nodes, as described in Materials and Methods (one of three experiments). As controls, purified human T cells without dendritic cells were activated with immobilized anti-CD3 + anti-CD28 antibodies (one of three experiments).

production of kynurenine from tryptophan and (*b*) a biological readout measured as the ability to prevent the suppression of T-cell proliferation caused by IDO-expressing dendritic cells.

Figure 3*A* shows enzyme kinetics (kynurenine production) using recombinant human IDO enzyme in a cell-free assay system. Using the recombinant enzyme, the L isomer of 1-methyl-tryptophan functioned as a competitive inhibitor (K_i = 19 μmol/L), whereas the D isomer was much less effective (no K_i found at 1-methyl-tryptophan concentrations up to 100 μmol/L). The DL mixture was intermediate, with a K_i of 35 μmol/L. These values are consistent with the published literature for studies using cell-free enzyme assays for IDO (37).

We next tested the different isomers in a biological assay, based on the intracellular IDO enzyme expressed by living cells (in this case, HeLa cells activated with IFN-γ, Fig. 3*B*). Kynurenine production by HeLa cells showed a pattern of inhibition similar to that of the cell-free recombinant enzyme, with L-1-methyl-tryptophan being more effective than D-1-methyl-tryptophan. In other studies (data not shown), similar results were obtained using the murine MC57 tumor cell line transfected with recombinant mouse IDO and also the simian COS cell line transfected with human IDO: in each of these transfected cell lines, L-1-methyl-tryptophan was superior to D-1-methyl-tryptophan at inhibiting kynurenine production.

In contrast to the behavior of cell lines, when primary human monocyte–derived dendritic cells were used as the IDO-expressing cells (Fig. 3*C*), the D isomer of 1-methyl-tryptophan was found to be at least as effective as the L isomer in its ability to inhibit IDO activity (measured as kynurenine production in culture supernatants). In these assays, dendritic cells were activated physiologically by exposure to T cells in allo-MLRs, rather than with recombinant IFNγ, because we have previously shown that IFNγ alone is not sufficient to activate functional IDO in dendritic cells prepared by this protocol (13, 14).

In addition to kynurenine production, we and others have shown that IDO suppresses proliferation of T cells responding to antigens presented by IDO$^+$ dendritic cells (13, 14, 22). Figure 3*D* shows a comparison of the different 1-methyl-tryptophan isomers on human T-cell proliferation in allo-MLRs stimulated by IDO$^+$ monocyte–derived dendritic cells (similar to the MLRs shown in Fig. 3*C*, but using T-cell proliferation as the readout). Using this readout, the D isomer was found to be reproducibly superior to either the L isomer or the DL mixture, typically eliciting a 2- to 3-fold greater maximum level of T-cell proliferation. A similar pattern was seen using murine T cells (Fig. 3*D*). For mice, allo-MLRs were done using IDO$^+$ dendritic cells isolated directly from murine tumor-draining lymph nodes, as previously described (22). These tumor-activated dendritic cells were used to present a constitutive allo-antigen to BM3 TCR-transgenic T cells (specific for the $H2K^b$ antigen expressed by the C57BL/6 dendritic cells). In this model, just as in the human system, the D isomer of 1-methyl-tryptophan was superior in supporting activation and proliferation of T cells, compared with either the L or DL forms.

To test for nonspecific (off-target) effects of 1-methyl-tryptophan on the T cells themselves, control experiments were done using purified human T cells stimulated by immobilized anti-CD3 + anti-CD28 antibodies (i.e., without any dendritic cells present to express IDO). Under these conditions, none of the 1-methyl-tryptophan preparations had any detectable effect on T-cell proliferation (Fig. 3*D*). Additional studies (shown in Supplementary Fig. S1) were done further evaluating the D isomer, using MLRs stimulated by dendritic cells derived from mice with a targeted disruption of the *IDO* gene (IDO-KO mice). MLRs using IDO-KO dendritic cells showed that the effects of the D isomer were completely lost when the stimulating dendritic cells lacked IDO. Thus, the D isomer of 1-methyl-tryptophan exerted its effects in MLR specifically by targeting the *IDO* gene expressed by the dendritic cells, not through an off-target effect.

Western blots suggest the possible existence of more than one isoform of IDO. The cell type–specific effects of the different isomers of 1-methyl-tryptophan prompted us to ask whether there might be more than one form of IDO expressed in different cells. Published databases suggested potential alternate splicing isoforms of human IDO differing primarily in the COOH-terminal portion of the molecule.[8] Therefore, we generated polyclonal antibodies against peptide sequences in the NH_2-terminal and COOH-terminal portions of the IDO molecule for use in Western blots, as described in the Supplementary Material.

Figure 4*A* shows Western blots using the two different antibodies. Samples were prepared from human monocyte–derived macrophages, as a known source of IFNγ-inducible IDO (11). As shown in Fig. 4*A*, the NH_2-terminal antibody detected a band of ~44 kDa, which was present both before and after IFNγ stimulation, and which showed little apparent change with IFNγ. In contrast, the COOH-terminal antibody detected an antigen of ~42 kDa, which was only visible after IFNγ treatment. A similar pattern of two different constitutive and inducible bands has been described for IDO expression by in other cell types (42). We and others have also shown that IDO can be expressed constitutively at the protein level (e.g., as with the higher molecular weight band) without necessarily showing enzymatic activity until activated (13, 43). In other experiments (data not shown), HeLa cells showed the same pattern of bands and the same response to IFNγ, as did the monocyte-derived macrophages in Fig. 4*A*.

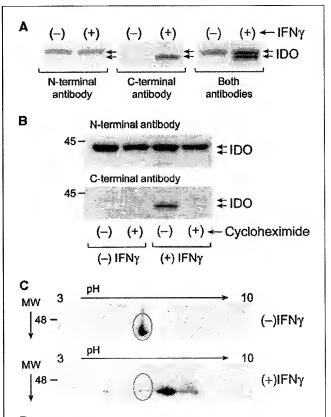


Figure 4. Evidence for two possible isoforms of human IDO. *A*, human monocyte–derived macrophages were prepared as described (11), with or without IFNγ treatment for the final 24 h. Lysates were analyzed by Western blot using antibodies against the NH_2-terminal portion of IDO, the COOH-terminal portion, or a mixture of the two antibodies. All blots were stripped and reprobed for β-actin (data not shown) to confirm even loading. *B*, macrophages, as above, were treated with or without IFNγ, in the presence or absence of cycloheximide (10 μg/mL). β-Actin blots (data not shown) confirmed even loading. *C*, lysates of macrophages with and without IFNγ pretreatment were analyzed by two-dimensional electrophoresis, followed by Western blotting with the NH_2-terminal–specific anti-IDO antibody. *D*, human monocyte–derived dendritic cells were cultured for 7 d as described in Materials and Methods, with or without addition of a maturation cocktail during the final 48 h. IFNγ was added during the last 24 h. Western blots were done as in (*B*), with the same blot stripped and reprobed for each anti-IDO antibody and the β-actin loading control.

[8] J. Thierry-Mieg et al. AceView: identification and functional annotation of cDNA-supported genes in higher organisms—*Homo sapiens* gene INDO, encoding indole-amine-pyrrole 2,3 dioxygenase. Available from http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly.

Figure 4*B* shows that expression of the IFN-γ-inducible (lower molecular weight, COOH-terminal) band was blocked by cycloheximide, suggesting that it represented a newly synthesized protein, rather than a posttranslational modification of the larger isoform. Although conventional Western blot analysis did not reveal any obvious change in the larger molecular weight (NH_2-terminal) isoform in response to IFN-γ, two-dimensional Western blots (Fig. 4*C*) revealed that there was a significant IFN-γ-induced shift in isoelectric point (up to 2 pH units). Thus, these data revealed that both forms of IDO were in fact IFN-γ responsive, with the larger form appearing to undergo some IFN-induced posttranslational modification, whereas the smaller form seemed to be synthesized *de novo*.

Regulation of IDO activity in dendritic cells is more complex than in macrophages, with multiple factors reported to influence both protein expression and enzymatic activity (17, 19). When we analyzed human monocyte–derived dendritic cells by Western blot (Fig. 4*D*), there was significant up-regulation of the larger (NH_2-terminal) isoform with dendritic cell maturation, whereas IFN-γ treatment had no discernible effect on this band in dendritic cells. The smaller (COOH-terminal) isoform showed no expression in immature dendritic cells and was not inducible in dendritic cells by IFN-γ. However, the COOH-terminal isoform underwent marked up-regulation with dendritic cell maturation (again independent of IFN-γ). Thus, the regulation of the two IDO isoforms in dendritic cells was complex and differed from their regulation in macrophages. However, the essential point was similar for dendritic cells: that more than one species of IDO was present, and that the pattern of expression was regulated by biologically relevant cytokine signals.

Efficacy of the D isomer of 1-methyl-tryptophan in chemoimmunotherapy. Based on the superiority of the D isomer in supporting T-cell activation *in vitro*, we tested the D isomer of 1-methyl-tryptophan *in vivo* using the B16F10 model. Established (day 7) B16F10 tumors were treated with cyclophosphamide plus D-1-methyl-tryptophan in a design similar to Fig. 1*A*. However, in these studies, the dose of the D isomer was reduced 4-fold compared with the dose of the DL mixture used in Fig. 1*A*, based on its superior efficacy *in vitro*. Even at the lower dose, D-1-methyl-tryptophan + cyclophosphamide showed significant growth delay compared with cyclophosphamide alone (Fig. 5*A*). Similar results were seen with a second chemotherapeutic agent gemcitabine (Fig. 5*B*). Neither gemcitabine alone nor D-1-methyl-tryptophan alone had a significant effect on B16F10 tumor growth, but together, the combination produced a significant growth delay.

D-1-methyl-tryptophan had no effect on B16F10 tumors when used as a single agent, but B16F10 is not a highly immunogenic tumor; we therefore asked whether D-1-methyl-tryptophan alone might show an effect if a more immunogenic tumor was used. B78H1-GM-CSF is a subline of B16 that has been transfected with granulocyte macrophage colony-stimulating factor (GM-CSF) to increase recruitment of APCs to the tumor and draining lymph nodes (44). The tumor is modestly immunogenic, although if implanted without irradiation, the tumors invariably grow and kill the host (45). In this somewhat more immunogenic model, D-1-methyl-tryptophan, as a single agent, was found to have a modest but reproducible and statistically significant effect on the growth (Fig. 5*C*, *left*). This modest antitumor effect was lost when the hosts were immunodeficient Rag1-KO mice (Fig. 5*C*, *middle*), showing that the effect of D-1-methyl-tryptophan was immune mediated. Likewise, the effect of D-1-methyl-tryptophan was lost when the less immunogenic parental tumor (without GM-CSF) was used in place of B78H1-GM-CSF (Fig. 5*C*, *right*). Thus, D-1-methyl-tryptophan did show some modest effect as a single agent when used with an artificially immunogenic tumor. However, this was substantially less potent than the effect of 1-methyl-tryptophan in combination with chemotherapy.

Comparison of D versus L isomers in chemo-immunotherapy. We next did side-by-side comparisons of the different isomers of 1-methyl-tryptophan in chemo-immunotherapy regimens. Figure 6*A* shows a comparison of D versus L versus DL forms of 1-methyl-tryptophan in orthotopic 4T1-luc tumors. Each 1-methyl-tryptophan preparation was given in combination with low-dose cyclophosphamide (25 mg/kg/dose by oral gavage once per week). Although minor effects were observed with the other combinations, only D-1-methyl-tryptophan with cyclophosphamide showed a statistically significant prolongation of survival relative to cyclophosphamide alone (for clarity, these two groups are re-graphed together in the second plot). A second, similar experiment showed the same results, reproducing the survival advantage of D-1-methyl-tryptophan over L-1-methyl-tryptophan in combination with cyclophosphamide.

Figure 6*B* compares the D versus L isomers of 1-methyl-tryptophan in the autochthonous MMTV-*Neu* breast tumor model. Both isomers were delivered orally for 5 days, as in Fig. 2*C*, in combination with paclitaxel. In this model also, D-1-methyl-tryptophan was found to be superior to L-1-methyl-tryptophan (in these studies, the L isomer showed no effect compared with chemotherapy alone).

Specificity of the D isomer for host IDO *in vivo*. Finally, one critical outstanding question was the target specificity of the D isomer *in vivo*. We had shown in Supplementary Fig. S1 (Supplementary Material) that the D isomer of 1-methyl-tryptophan specifically targeted the *IDO* gene *in vitro*. However, it was possible that *in vivo*, D-1-methyl-tryptophan might exert an antitumor effect via some other off-target mechanism. Figure 6*C* addresses this question by comparing tumors grown in wild-type (IDO sufficient) mice versus tumors grown in IDO-KO mice, each treated with cyclophosphamide + D-1-methyl-tryptophan. The tumors that grew in the IDO-KO hosts would, by definition, have been selected for their lack of dependence on IDO (i.e., they must necessarily be escape variants that could grow in the absence of IDO). Thus, if D-1-methyl-tryptophan truly targeted IDO, then treating tumors grown in IDO-KO mice with D-1-methyl-tryptophan should have no effect on tumor growth; conversely, if D-1-methyl-tryptophan was not specific for IDO, then any off-target effects should be retained in the IDO-KO hosts. Figure 6*C* shows that tumors grown in IDO-KO mice became completely refractory to the effects of D-1-methyl-tryptophan, thus confirming that IDO was the target of D-1-methyl-tryptophan *in vivo*, as hypothesized. More specifically, these studies suggested that in this model, the relevant target for D-1-methyl-tryptophan was IDO expressed by host cells, rather than by tumor cells, because the tumor cells were the same in both cases.

Discussion

In the current study, we show significant differences in biological activity between the D and L stereoisomers of 1-methyl-tryptophan. The L isomer was superior at inhibiting activity of purified recombinant IDO enzyme in a cell-free assay and also at inhibiting IDO enzymatic activity in HeLa cells and other cell lines. In

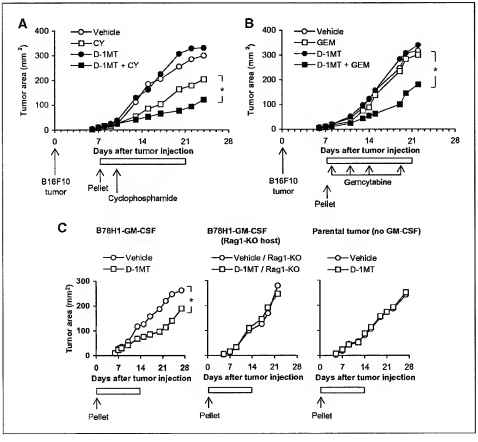


Figure 5. Effect of parenteral D-1-methyl-tryptophan in the B16F10 model. *A*, mice with B16F10 tumors were treated in a design similar to Fig. 1*A*, except using the D isomer of 1-methyl-tryptophan at a 4-fold lower dose (5 mg/d by timed release pellets). Cyclophosphamide was given at 150 mg/kg i.p. Three identical experiments were pooled and analyzed by ANOVA. *, $P \leq 0.05$. *B*, experimental design similar to (*A*), using gemcitabine 120 mg/kg i.p. on days 8, 11, 14, and 19 following B16F10 tumor implantation. Three experiments were pooled and analyzed by ANOVA. *, $P < 0.05$. *C*, B78H1-GM-CSF tumors, or parental tumors without the GM-CSF transgene, were implanted as indicated. Beginning at the time of implantation, mice received 14-day pellets of D-1-methyl-tryptophan (5 mg/d) or vehicle control. *Left*, three experiments were pooled and analyzed by ANOVA. *, $P = 0.011$. *Middle*, all hosts were Rag1-KO. *Right*, tumors lacked the GM-CSF transgene (neither of these groups showed significant differences).

contrast, the D isomer was at least as effective as the L isomer at inhibiting IDO enzymatic activity expressed by human or mouse dendritic cells. Unexpectedly, the D isomer was found to be significantly superior to both the L form and the DL mixture when tested by the biologically important readout of T-cell activation in MLRs. *In vivo*, a head-to-head comparison of the antitumor effect of the two isomers showed that the D isomer was more effective than the L isomer, using two different tumors and different chemoimmunotherapy regimens. Thus, the *in vitro* superiority of the D isomer for enhancing T-cell activation in MLRs seemed to correctly predict the superior *in vivo* antitumor efficacy in the models tested, whereas the results of the cell-free enzyme assays did not.

The superiority of the L isomer in the cell-free enzyme assay was expected from the literature (37). However, to our knowledge, no comparison of the two isomers of 1-methyl-tryptophan has been previously reported using assays based on intact cells. Such cell-based systems are important because different cell types may respond differently to the two isomers, as we have now shown. The molecular basis for these cell type–specific differences is not yet known. Possibilities include differential transport into or out of the cells, different subcellular compartmentalization of the inhibitors, or altered metabolism by cellular enzymes. It is also possible that there may be different isoforms of IDO (as could be suggested by our Western blot data), and these might have different sensitivities to the two isomers, although this is currently speculative. Finally, it may be that 1-methyl-tryptophan exerts some of its inhibitory effects on IDO not by competing directly for the catalytic site but by altering enzyme activity in another way that does not register in the cell-free enzyme assay.

Others have also reported efficacy of the D isomer of 1-methyl-tryptophan for enhancing T-cell responses *in vitro* and *in vivo* (46, 47). Importantly, our data unambiguously showed that the T cell–enhancing effect of D-1-methyl-tryptophan *in vitro* was completely lost when APCs were derived from IDO-KO mice; and, likewise, the antitumor efficacy of D-1-methyl-tryptophan *in vivo* was lost when the tumor-bearing hosts were IDO-KO. Thus, the molecular target of D-1-methyl-tryptophan was indeed IDO, and the efficacy of D-1-methyl-tryptophan was not due to some off-target effect. This would also be consistent with recent studies using RNA–knock-down techniques, which concluded that the major molecular target of the DL-mixture of 1-methyl-tryptophan was IDO, rather than an off-target effect (48).

One critical reason underlying the superior activity of the D isomer *in vivo* may be our observation that the L isomer seemed actively inhibitory for T-cell activation in MLRs. Both isomers were equally effective at blocking the enzymatic activity of IDO in MLRs (measured as kynurenine production in the supernatant); yet, the L isomer could not produce the same high levels of T-cell proliferation achieved by the D isomer. Revealingly, the DL mixture also proved less effective than the D isomer alone, suggesting that the presence of the L isomer actively inhibited T-cell proliferation. The nature of this inhibition is currently unknown. However, it did not seem to be due to a direct toxic effect of L-1-methyl-tryptophan on the T cells themselves because T cells stimulated by mitogen (i.e., in the absence of IDO-expressing dendritic cells) were no longer affected by L-1-methyl-tryptophan. This suggests that the off-target inhibitory effect of the L isomer might be due to a toxic effect of L-1-methyl-tryptophan on the IDO-expressing dendritic cell itself

(e.g., rendering it less able to present antigen to the T cells). Perhaps consistent with such an off-target effect on dendritic cells, it has recently been reported that exposure of dendritic cells *in vitro* to the DL-mixture of 1-methyl-tryptophan at 1,000 μmol/L (much higher than the maximum concentration used in the current study) caused alteration in dendritic cell function, which did not seem related to the effect of DL-1-methyl-tryptophan on IDO itself (49). Alternatively, the T cells might be sensitive to some metabolite of the L isomer generated by the dendritic cells. In either case, it seems that the D isomer of 1-methyl-tryptophan escaped this off-target inhibitory effect on T-cell activation, perhaps precisely because it was not the "natural" stereoisomer.

Although the D isomer showed superior efficacy in our chemo-immunotherapy models, the L isomer proved better at inhibiting IDO in HeLa cells and in mouse tumor cell lines transfected with IDO. Thus, it may be that in certain biological contexts the L isomer

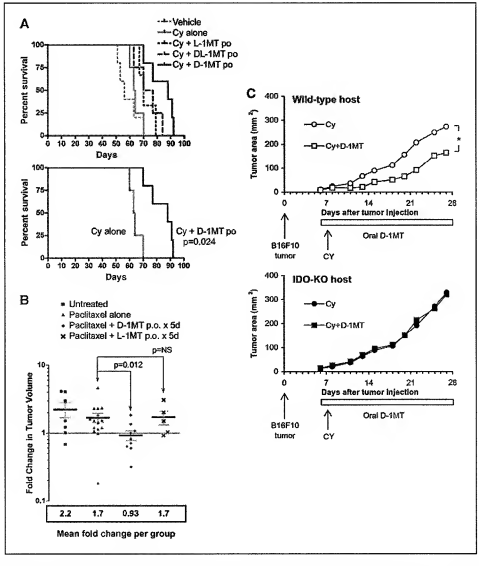


Figure 6. D-1-methyl-tryptophan provides greater survival benefit in combination therapy, in an IDO-dependent fashion. *A*, 4T1-luc orthotopic isografts were established in the mammary fat pad. Cyclophosphamide was given at 25 mg/kg orally once a week, and 1-methyl-tryptophan (D, L, or DL) given at 400 mg/kg by oral gavage twice daily, five times a week by gavage, beginning at the time of tumor implantation. *Top*, time to endpoint for all groups; *bottom*, only the cyclophosphamide versus cyclophosphamide + D-1-methyl-tryptophan groups, for clarity. The comparisons of interest were between D-1-methyl-tryptophan + cyclophosphamide versus cyclophosphamide and L-1-methyl-tryptophan + cyclophosphamide versus cyclophosphamide. Because survival data were not censored, groups were analyzed using a two-group Wilcoxon exact test; statistical significance was determined at $P < 0.025$. The combination of D-1-methyl-tryptophan + cyclophosphamide showed a significant survival benefit over cyclophosphamide alone ($P = 0.024$), whereas L-1-methyl-tryptophan + cyclophosphamide was not different from cyclophosphamide alone ($P = 0.14$). *B*, MMTV-*Neu* mice with tumors were treated for 2 wks as in Fig. 2*B*, receiving either vehicle alone, paclitaxel alone, or paclitaxel (13.3 mg/kg q. MWF) plus oral D-1-methyl-tryptophan or L-1-methyl-tryptophan for 5 d, as indicated. For statistical analysis, the comparisons of interest were D-1-methyl-tryptophan + paclitaxel versus paclitaxel alone and L-1-methyl-tryptophan + paclitaxel versus paclitaxel alone. Significance was determined at $P < 0.025$ using a two-group Wilcoxon exact test. The fold change of the D-1-methyl-tryptophan + paclitaxel group was significantly smaller than that of paclitaxel alone ($P = 0.012$), whereas paclitaxel + L-1-methyl-tryptophan was not different from paclitaxel alone ($P = 0.85$). *C*, effects of the D isomer of 1-methyl-tryptophan require an intact host *IDO* gene. B16F10 tumors were grown in either wild-type B6 hosts or IDO-KO hosts on the B6 background. All groups received cyclophosphamide, with or without oral D-1-methyl-tryptophan (2 mg/mL in drinking water). Analysis by ANOVA showed that cyclophosphamide + D-1-methyl-tryptophan was significantly different (*, $P < 0.05$) than cyclophosphamide alone for the wild-type hosts, but there was no effect of D-1-methyl-tryptophan when tumors were grown in IDO-KO hosts.

might be preferable, whereas in other contexts, the D isomer is superior. This might become relevant where the target of 1-methyl-tryptophan is IDO expressed by the tumor cells themselves, rather than by host dendritic cells. However, the data from our *in vitro* T-cell activation models and from our *in vivo* chemo-immunotherapy models suggest that in these systems, the beneficial effect of the D isomer on T-cell activation is the key advantage, rendering the D isomer superior in these settings. Furthermore, based on the fact that efficacy of D-1-methyl-tryptophan was lost when the host mice were genetically deficient in IDO (Fig. 6C), our data suggest that the molecular target of D-1-methyl-tryptophan in our system was the IDO activity expressed specifically by host APCs, not by the tumor cells themselves.

In the murine models used in this study, relatively high doses of 1-methyl-tryptophan were required to see an antitumor effect. However, this seems to represent a peculiarity of 1-methyl-tryptophan pharmacokinetics in mice. Preclinical pharmacology studies in both rats and canines (to be published elsewhere) show that these animals require significantly lower doses per kilogram to achieve plasma levels in the same range. These lower doses should be readily achievable clinically.

The combination of 1-methyl-tryptophan with chemotherapy (cyclophosphamide, paclitaxel or gemcitabine) was more potent against established tumors than either 1-methyl-tryptophan or chemotherapy alone. Regimens featuring chemotherapy plus immunotherapy are receiving increasing attention (34, 35). In part, this is because they are readily applicable in the clinic because patients do not have to be denied standard chemotherapeutic agents to receive immunotherapy. In addition, there is a sound mechanistic rationale underlying combined chemo-immunotherapy. Chemotherapy causes death of tumor cells, thus releasing tumor antigens into the host antigen-presentation pathway (34). In addition, certain chemotherapy drugs seem to decrease the number and activity of regulatory T cells (50, 51), which may assist the immunotherapy regimens in breaking tolerance to tumor antigens. Finally, the recovery phase from chemotherapy-induced lymphopenia seems to constitute a favorable window for reactivating previously tolerized T cells (41). However, despite these effects, chemotherapy alone does not elicit an effective antitumor immune response. We hypothesize that one reason for this failure is because the antigens released by chemotherapy are presented first in the tumor-draining lymph nodes. We and others have previously shown that tumor-draining lymph nodes are a highly tolerogenic microenvironment (52), due at least in part to the presence of IDO-expressing APCs (22, 25). Thus, IDO$^+$ host APCs may play an important pathogenic role in helping the tumor re-establish immunologic tolerance toward itself after it is disrupted by chemotherapy. Based on our current data, we hypothesize that the addition of an IDO inhibitor drug during this post-chemotherapy period may allow the tumor-bearing host to mount an effective immune response to tumor antigens during this post-chemotherapy window of opportunity.

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Exhibit B

Modulation of tryptophan catabolism by human leukemic cells results in the conversion of $CD25^-$ into $CD25^+$ T regulatory cells

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Indoleamine 2,3-dioxygenase (IDO) is a novel immunosuppressive agent expressed in some subsets of normal and neoplastic cells, including acute myeloid leukemia (AML) cells. Here, we show that IDO expression correlates with increased circulating $CD4^+CD25^+$ *FOXP3*$^+$ T cells in patients with AML at diagnosis. In vitro, IDO^+ AML cells increase the number of $CD4^+$ $CD25^+$ T cells expressing surface CTLA-4 and *FOXP3* mRNA, and this effect is completely abrogated by the IDO inhibitor, 1-methyl tryptophan (1-MT). Purified $CD4^+CD25^+$ T cells obtained from coculture with IDO^+ AML cells act as T regulatory (T_{reg}) cells because they do not proliferate, do not produce interleukin (IL)–2, and inhibit naive T-cell proliferation. Coculture with IDO^+AML cells results in the conversion of $CD4^+CD25^-$ into $CD4^+CD25^+$ T cells, which is completely abrogated by 1-MT. Moreover, in mice, intrasplenic injection of IDO^+ leukemia/lymphoma A20 cells induces the expansion of bona fide T_{reg} cells by conversion of $CD4^+CD25^-$ T cells; this effect is counteracted by 1-MT treatment. These data indicate that AML cells induce T-cell tolerance by directly converting $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T_{reg} cells through an IDO-dependent mechanism. (Blood. 2007;109:2871-2877)

Introduction

Indoleamine 2,3-dioxygenase (IDO) is a key enzyme in the tryptophan metabolism that catalyzes the initial rate-limiting step of tryptophan degradation along the kynurenine pathway.[1] Tryptophan starvation by IDO consumption inhibits T-cell activation,[1,2] while products of tryptophan catabolism, such as kynurenine derivatives and O_2-free radicals, regulate T-cell proliferation and survival.[1,3] Thus, IDO has been shown to exert an immunosuppressive activity, and cell populations, including regulatory dendritic cells (DCs) and bone marrow (BM)–derived mesenchymal stem cells (MSCs), expressing IDO have the capacity to suppress T-cell responses to auto- and alloantigens.[4,5]

A wide variety of human solid tumors express IDO.[6] More recently, we demonstrated that also acute myeloid leukemia (AML) cells, but not their normal counterparts (ie, $CD34^+$ hematopoietic stem/progenitor cells [HSCs]), express an active IDO protein, which converts tryptophan into kynurenine and inhibits allogeneic T-cell proliferation.[7]

Naturally arising $CD4^+CD25^+$ $Foxp3^+$ T regulatory (T_{reg}) cells are known to suppress most types of immune response,[8,9] including antitumor immunity.[10-13] IDO is expressed and is functionally active in placenta, which, in turn, is infiltrated by $CD4^+CD25^+$ T_{reg} cells.[14-16] Moreover, *Candida albicans* infection increases the number of T_{reg} cells because of IDO induction in host antigen-presenting cells (APCs).[17] In human cancers, tumor-draining lymph nodes contain IDO-expressing DCs that enhance T_{reg} cell function.[18] These data suggest the close relationship between IDO activity and the occurrence of T_{reg} cells,[19] but the mechanism governing the generation of T_{reg} cells by IDO-expressing tumors is presently unknown.

In the present study, we investigated whether the expression of IDO by AML cells may play a direct role in the development of T_{reg} cells.

Materials and methods

Cells

All human samples were obtained after informed consent was signed, according to institutional guidelines. Approval was obtained from Bologna Hospital Ethical Committee. Buffy coats were obtained from healthy adults during the preparation of transfusion products. BM and/or peripheral blood (PB) samples including at least 70% leukemic cells were harvested from 76 patients with AML at diagnosis. $CD3^+$ and $CD4^+$ cells were purified from the mononuclear cell (MNC) fraction by MiniMacs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions (purity of $CD3^+$ and $CD4^+$ cell populations was always greater than 95%). MSCs were generated from BM cells as previously reported.[20]

Murine A20 and CT26 cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) or MEM (Whittaker Bioproducts) supplemented with 10% FCS (Sera Lab, Crawley Down, United

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Kingdom), penicillin/streptomycin (50 U/mL), L-glutamine (2 mM), HEPES buffer (Whittaker Bioproducts), and nonessential amino acids (Whittaker Bioproducts), hereafter referred to as complete medium.

RT-PCR

Polymerase chain reaction (PCR) was performed on cDNA as described elsewhere.[21] Human INDO: forward 5′-ATGTGTGGGGCAAAGGT-CATGG-3′, reverse 5′-AAGTGTCCCGTTCTTGCATTTGC-3′; mouse INDO: forward 5′-GTCTGCTGTATGAGGGGGTCT-3′, reverse 5′-CATTTGAGGGCTCTTCCGACTTG-3′; and human Foxp3: forward 5′-CCCACTTACAGGCACTCCTC-3′, reverse 5′-CTTCTCCTTCTCCAG-CACCA-3′. As internal control, human β_2-microglobulin (β_2M) and mouse glyceraldehyde-3-phosphate dehydrogenase (Gapdh) genes were amplified. PCR products were separated and visualized on 2% agarose gel stained with ethidium bromide.

IDO expression and activity

Human primary AML cells as well as murine cell lines were tested for IDO expression both at the mRNA and protein levels. PCR analysis of human IDO was performed as described. For detection of IDO protein, mouse anti-IDO monoclonal antibody (mAb) (clone 10.1; Chemicon, Temecula, CA) and mouse IgG isotype antibody (Pharmingen, San Diego, CA) were used. Immunocytochemistry analysis was performed on cytospins, as previously reported.[22] For IDO activity, the amount of L-tryptophan and L-kynurenine in culture supernatant was measured by high-performance liquid chromatography (HPLC) using a reverse-phase column as previously reported with modifications.[4] Cells (10×10^6/well) were cultured in complete medium, and supernatants were collected after 72 hours. After adding N-acetyl-tryptophan and 3-nitro-L-tyrosine (50 μM final concentration), as internal standard for L-tryptophan and L-kynurenine, respectively, 100 μL of supernatant was injected into a C-18 column. The absorbance of column effluent was monitored with the UV detector at a wavelength of 270 nm for tryptophan and 360 nm for kynurenine. The calibration curve for the quantitative analysis was performed injecting the standard molecule at 6 different concentrations between 0 and 100 μM for tryptophan and between 0 and 12.5 μM for kynurenine.

Purification of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ subsets

CD4$^-$CD25$^+$ and CD4$^+$CD25$^-$ cells were isolated by MiniMacs CD4$^+$CD25$^+$ regulatory T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. To achieve highest purity, positive and negative cell fractions were separated over a second column. CD4$^+$CD25$^+$ cells obtained in the positive fraction were routinely more than 90% of total cells as evaluated by fluorescence-activated cell sorting (FACS) analysis. CD4$^+$CD25$^-$ T cells accounted for more than 98% of the cells collected in the negative fraction. Purified T cells were used for phenotypic and functional assays. As positive control sample for CD4$^+$CD25$^+$ T cells, total MNCs were stimulated for 48 hours with mAbs against CD3 and CD28 (Pharmingen, San Diego, CA).

In vitro T-cell culture with AML cells

AML cells (1×10^5/mL) were cultured in RPMI complete medium with 10^6/mL allogeneic CD3$^+$ T cells/mL for 7 days in the presence and absence of optimal concentrations (1000 μM) of the IDO inhibitor 1-methyl-D-tryptophan (1-MT; Sigma-Aldrich, St Louis, MO), as evaluated in preliminary experiments.[23] When indicated, total CD3$^+$, CD4$^-$CD25$^+$, and CD4$^+$CD25$^-$ cells were labeled with CFSE (Molecular Probes, Eugene, OR) by incubation with 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) in PBS containing 5% FCS for 5 minutes at 37°C and then used as responders. At the end of culture, cells were collected and used for phenotypic and functional assays. After 24, 48, 72, and 96 hours of culture, T cells were tested for apoptosis by using human Annexin-V and propidium iodide (Bender Medsystems, Burlingame, CA).

Conditioned medium experiments

IDO$^-$ and IDO$^-$ AML cells (10×10^6/mL) were cultured in complete medium RPMI 1640 (Whittaker Bioproducts) containing 25 μM L-tryptophan in the presence and absence of 1-MT (1000 μM). In selected experiments, L-tryptophan (Sigma-Aldrich) was added to culture medium to a final concentration of 150 μM. After 72 hours, supernatants (conditioned media) were collected and tested by HPLC for L-tryptophan and L-kynurenine concentrations. T cells (10×10^6/mL) were cultured in AML-derived conditioned medium with 10^5/mL IDO$^-$ AML cells for 7 days. At the end of culture, T cells were collected and used for flow cytometry analysis.

T-cell proliferation and in vitro suppression assays

Standard allogenic mixed lymphocyte reaction (MLR) was performed as previously described.[21] Briefly, naive and leukemic cell-cultured CD3$^+$ cells (10^5/well) were incubated with different numbers of irradiated (3000 cGy) stimulators for 5 days. Then, cells were pulsed with 1 μCi (0.037 MBq) per well of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) and tested as previously described.[21] The stimulation index (SI) was calculated for each individual experiment as follows: SI = cpm (counts per minute) (T-cell responders + stimulators)/cpm (T-cell responders).

To test their suppressive activity, control naive CD3$^+$ cells and leukemic cell-cultured T-cell subsets were added to cultures consisting of the same donor-derived naive CD3$^+$ T cells (5×10^4/well) as responders, and the same number of irradiated allogeneic T-cell depleted MNCs (APCs) as stimulators. After 5 days, cultures were pulsed with 1 μCi (0.037 MBq) per well of [^{3}H]thymidine and tested as previously described.[21]

Experimental T-cell proliferation was compared with that observed in the presence of control T cells and expressed as a percentage of inhibition.

In vivo experiments

BALB/c mice (8-10 weeks old) were obtained from Charles River (Calco, Italy) and maintained at the Istituto Nazionale Tumori under standard conditions according to institutional guidelines. Female mice were injected intraspleni cally with 10^5 A20 cells or with PBS. Mice received 1-D and L-MT (Sigma-Aldrich) in the drinking water (3.5 mL/day) at the final concentration of 5 mg/mL.[6] After the injection (25 days), mice were killed, and the percentage of CD4$^+$CD25$^+$ T cells was evaluated in the spleen, pooled lymph nodes, and thymus. Intracellular staining of Foxp3 (FJK-16S) was performed on purified CD4$^+$CD25$^+$ and CD4$^+$ CD25$^-$ according to the manufacturer's instructions (e-Bioscience, San Diego, CA).

To test T_{reg} cell suppressive activity, 5×10^4 CD4$^+$CD25$^-$ were cultured with 5×10^4 accessory cells (ACs) consisting in the whole 3Gy-irradiated spleen, with or without T_{reg} cells at the indicated ratio, for 72 hours in complete medium. Anti-CD3 (1 μg/mL; eBioscience) was added to each well for stimulation. [^{3}H] thymidine (1 μCi [0.037 MBq]/well; Amersham Pharmacia Biotech) was added for the last 10 hours of culture and measured in a microplate scintillator counter (Tomtec; Wallac, Turku, Filland). To study in vivo conversion, 10×10^6 Thy1.1-derived CD4$^+$CD25$^-$ cells, previously labeled with 5 μM CFSE for 15 minutes at 37°C, were transferred by tail-vein injection into recipient mice that had been inoculated intraspleni cally 10 days before with 10^5 A20 cells or subcutaneously with 5×10^4 CT26 cells. The percentage of converted CD4$^+$CD25$^-$ cells in the spleen was assessed after 10 days. Cells were stained with phycoerythrin (PE)–conjugated anti-CD25, PE-Cy5, anti-CD4, and APC-Thy1.1 antibodies. The percentage of CD25$^+$ cells over CFSE$^+$ cells was calculated on gated Thy1.1$^+$ CD4$^+$ cells.

Immunophenotype studies

Dual-color immunofluorescence was performed using the following panel of mAbs: PE- or fluorescein isothiocyanate (FITC)–conjugated human anti-CD3 (clone UCHT1; Pharmingen); PE- or FITC-conjugated human anti-CD4 (clone RPA-T4; Pharmingen); PE- or FITC-conjugated human anti-CD8 (clone HIT8a; Pharmingen); PE-conjugated human anti-CD25 (clone M-A251; Pharmingen); FITC-conjugated human anti–cytotoxic T lymphocyte–associated antigen (anti–CTLA-4, clone CBL 591F; Cymbus Biotechnology, Hampshire, United Kingdom); FITC-conjugated human anti–HLA-DR (clone L242; Pharmingen); PE- or FITC-conjugated

anti–human CD45RO (clone UCHL-1; Pharmingen); and PE- or FITC-conjugated anti–human CD62L (clone MEL-14; Pharmingen). Negative controls were isotype-matched irrelevant mAbs (Pharmingen). FITC-conjugated anti–mouse CD4 (L3T4), PE-conjugated anti–mouse CD25 (PC61), and the relative isotype controls were all purchased from BD Bioscience (San Diego, CA). Cells were analyzed by using FACScan equipment (Becton Dickinson). A minimum of 10 000 events was collected in list mode on FACScan software.

Statistical analysis

Results are expressed as means plus or minus standard deviation (SD). Where indicated, differences were compared using the Student *t* test and χ-square analysis.

Results

In vivo correlation of IDO expression with increased CD4$^+$CD25$^+$ Foxp3$^+$ T cells

Primary AML samples (n=76) collected at diagnosis were tested for IDO expression. Of these 76 patients with AML, 40 (52%) were positive for both IDO mRNA and protein. Moreover, IDO protein was capable to convert tryptophan into kynurenine and to reduce T-cell alloreactivity (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).[7]

To test whether IDO expression in patients with AML correlates, in vivo, with the frequency of T_{reg} cells, PB samples were collected at diagnosis from patients with AML whose circulating T cells were assessable (9 IDO$^+$ and 12 IDO$^-$) and CD25 expression on gated CD4$^+$ cells was evaluated. As shown in Figure 1A, the percentage of CD4$^+$CD25$^+$ cells was significantly increased in IDO$^+$ AML samples compared with IDO$^-$ patients or healthy donors ($P = .03$). The T_{reg} nature of CD4$^+$CD25$^+$ cells was evaluated by testing the expression of the winged-helix/forkhead transcription factor FOXP3, which is a widely accepted marker for naturally occurring T_{reg} cells.[24] In particular, highly purified CD4$^+$ T cells were obtained from the PB of patients with AML and then evaluated for FOXP3 mRNA expression. Figure 1B shows that IDO expression correlates with FOXP3 reverse transcription (RT)–PCR positivity ($P = .002$). These data reveal a correlation between IDO expression and increased CD4$^+$CD25$^+$FOXP3$^+$ T-cell frequency in patients with AML at diagnosis.

IDO-expressing AML cells increase, in vitro, CD4$^+$CD25$^+$Foxp3$^+$ T cells

To investigate the role of IDO expression by AML cells on T_{reg} cell development, we cocultured IDO$^+$ and IDO$^-$ AML cells with highly purified allogeneic CD3$^+$ T cells, obtained from healthy donors, in the presence or absence of the IDO inhibitor 1-MT. The viability of cells cultured in presence of 1-MT was not different from that cultured in medium alone (79% ± 12% and 82% ± 16%, respectively), as well as CD4$^+$ and CD8$^+$ T-cell frequencies were not modified by the addition of 1-MT (data not shown). Coculture of T cells with IDO-expressing AML cells increased both the percentage of CD4$^+$CD25$^+$ T cells and the surface expression of CD25 (mean fluorescence intensity [MFI] 150 ± 45 and 980 ± 390 before and after coculture, respectively; Figure 2A-B; $P = .001$ and $P < .001$, respectively), whereas coincubation of T cells with IDO$^-$ AML cells had no effect on CD25 expression (Figure 2A-B). The addition of 1-MT to cocultures of T cells with IDO$^+$ AML cells restored the expression of CD25 to that observed before culture, whereas 1-MT had no effect on T cells cultured with IDO$^-$ AML cells (Figure 2A-B). CD4$^+$CD25$^+$ T cells cultured with IDO-expressing AML cells expressed CTLA-4, which was down-regulated in presence of 1-MT (Figure 2C) as well as HLA-DR, CD62L, and CD45RO (data not shown). The described pattern of surface markers suggests that CD4$^+$ T lymphocytes, after coculture with IDO-expressing AML cells, acquire a CD4$^+$CD25$^+$ T_{reg} cell phenotype, which was markedly inhibited in the presence of 1-MT. This conclusion was corroborated by showing that CD4$^+$CD25$^+$ but not CD4$^+$CD25$^-$ cells purified after coculture with IDO-expressing AML cells express FOXP3 mRNA (Figure 2D).

To investigate whether IDO activity in AML cells would result in the generation of a microenvironment that was capable per se to increase CD4$^-$CD25$^+$ T cells, a conditioned medium obtained from IDO$^+$ and IDO$^-$ AML cell cultures was used to stimulate naive CD3$^-$ T cells. Moreover, to test whether the availability of an excess of L-tryptophan within the conditioned medium could contrast IDO activity, resulting in a differential induction of CD4$^+$CD25$^-$ cells, the conditioned medium was generated both with standard (25 μM) and increased (150 μM) starting concentrations of L-tryptophan. According to IDO expression, only the conditioned medium collected from IDO$^+$ AML cells had decreased tryptophan and increased kynurenine concentrations (Figure 3A,C). When we compared the contents of conditioned mediums collected from IDO$^+$ AML cells previously cultured in 25 μM or 150 μM starting L-tryptophan, the amount of kynurenine

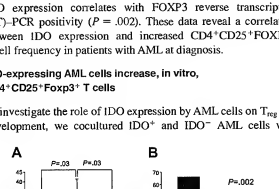


Figure 1. IDO expression correlates with increased CD4$^+$CD25$^+$Foxp3$^+$ T cells in patients with AML. (A) Percentage of circulating CD4$^+$CD25$^+$ T cells in PB collected from healthy donors and patients with AML at diagnosis. (B) Foxp3 mRNA expression in highly purified CD4$^-$ cells obtained from patients with AML. Data are expressed as a percentage of FOXP3$^+$ patients. Healthy donors, n = 9; IDO$^+$ patients, n = 9; and IDO$^-$ patients, n = 12. The data report the mean ± SD of independent experiments.

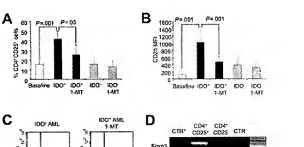


Figure 2. IDO expression in AML cells increased CD4$^+$CD25$^+$Foxp3$^+$ T cells. (A-B) Flow cytometry analysis of CD4$^+$CD25$^+$ T cells before and after culture with AML cells in the presence and absence of 1-MT (1000 μM). Data are expressed as the percentage of CD4$^+$CD25$^+$ T cells (A) and as CD25 MFI on gated CD4$^+$ T cells (B). Results are the mean ± SD of 10 independent experiments. (C) Surface CTLA-4 expression on purified CD4$^+$CD25$^+$ T cells obtained after culture with IDO-expressing AML cells in the presence and absence of 1-MT. Results are representative of 4 independent experiments. (D) FOXP3 mRNA expression by purified CD4$^+$CD25$^-$ and CD4$^+$CD25$^-$ T cells obtained after culture with IDO-expressing AML cells. Total MNCs stimulated with monoclonal antibodies against CD3 and CD28 were used as positive control. Results are representative of 4 independent experiments.

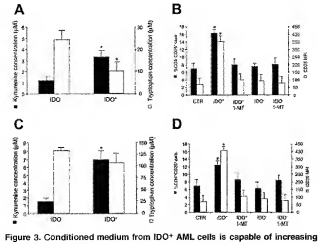


Figure 3. Conditioned medium from IDO$^+$ AML cells is capable of increasing CD4$^+$CD25$^+$ T cells. (A,C) HPLC assessment of L-tryptophan and L-kynurenine in the conditioned media obtained from IDO$^-$ and IDO$^+$ AML cells cultured with and without 1-MT (1000 μM). Starting L-tryptophan concentration was 25 μM (A) and 150 μM (C). *$P < .03$, IDO$^+$ versus IDO$^-$ cells. (B,D) CD3$^+$ T cells (10×10^6 cells/mL) were cultured with 10%mL IDO$^-$ AML cells in the conditioned media containing 25 μM (B) and 150 μM (D) starting L-tryptophan. At the end of culture, T cells were collected and used for flow cytomstry analysis of CD25 expression on gated CD4$^+$ cells. As control sample (CTR), noncultured CD3$^+$ T cells were used. *$P < .03$, IDO$^+$ versus CTR cells. The data report the mean ± SD of indepandant experiments.

was comparable, whereas final tryptophan concentration was much higher when 150 μM concentration was used. As shown in Figure 3B and 3D, culture of T cells in the conditioned medium obtained from IDO$^+$ AML cells resulted in the up-regulation of CD25 expression on CD4$^+$ T cells, and this effect was abrogated by adding 1-MT to AML cell cultures, whereas no effect on CD25 expression was observed when T cells were cultured in IDO$^-$ conditioned medium. Interestingly, similar results were observed with 25 and 150 μM starting L-tryptophan, indicating that tryptophan depletion from culture medium, which was minimal upon 150 μM concentration, does not play a major role in increasing CD4$^+$CD25$^+$ T cells. The conditioned medium experiments show that the microenvironment produced by IDO$^+$, but not by IDO$^-$, AML cells was capable per se to induce the expansion of CD4$^+$CD25$^+$ T cells.

These in vitro data demonstrate that tryptophan catabolism by IDO-expressing AML cells increases CD4$^+$CD25$^+$Foxp3$^+$ T cells.

AML-induced CD4$^+$CD25$^+$ T cells have regulatory activity

T cells obtained after primary coculture with IDO$^+$ AML blasts were compared with autologous naive counterparts for their ability to respond to allogeneic APCs in a secondary MLR. As shown in Figure 4A and 4B, total T cells showed reduced proliferation and IL-2 production ($P = .02$), which were completely restored by the addition of 1-MT to the same primary coculture. Moreover, when highly purified CD4$^+$CD25$^+$ T cells were used, almost no proliferation and barely detectable IL-2 production were observed (Figure 4A-B). Importantly, CD4$^+$CD25$^-$ T cells, which had been completely depleted of CD4$^+$CD25$^+$ cells, showed a marked increase in cell proliferation and IL-2 production compared with that of CD25$^+$ counterparts ($P = .001$), which was higher than that of naive, unpurified CD3$^+$ T cells. These data point to the functional activity of CD4$^+$CD25$^+$ T cells obtained after culture with IDO-expressing AML cells in reducing T-cell proliferation and IL-2 production.

An additional set of functional experiments was performed to validate the T_{reg} cell nature of the cells induced by IDO-expressing AML cells. Naive CD3$^+$ T cells were stimulated by allogeneic APCs in the presence of AML-cultured or naive autologous T cells. As shown in Figure 4C, T-cell proliferation was markedly reduced when total T cells previously cultured in the absence but not presence of 1-MT were used ($P = .02$). This effect was markedly increased when AML-cultured CD4$^-$CD25$^+$ T cells were added to cell cultures ($P = .03$), whereas CD4$^+$CD25$^-$ gave a similar effect to that of cells cultured with 1-MT (Figure 4C). The suppressive activity of CD4$^+$CD25$^+$ T cells obtained after culture with IDO-expressing AML cells was dose dependent (Figure 4D).

Taken together, these data support the hypothesis that CD4$^-$CD25$^-$ T cells induced by IDO-expressing AML cells retain immunosuppressive activity and may be considered bona fide T_{reg} cells.

AML cells convert CD4$^+$CD25$^-$ into CD4$^+$CD25$^+$ T cells through an IDO-dependent mechanism

Coculture of IDO-expressing AML cells with CD3$^+$ T cells resulted in the increase of CD4$^+$CD25$^+$ cells, which was paralleled by the decrease of CD4$^+$CD25$^-$ cells (Figure 5A; $P = .03$). This effect could be alternatively explained considering the expansion of CD4$^+$CD25$^+$ cells because of active proliferation, the increased tendency to undergo apoptosis of CD4$^+$CD25$^-$ over CD4$^-$CD25$^+$ T cells, or the conversion of CD4$^-$ CD25$^-$ into CD4$^+$CD25$^+$ T cells.

To address these points, CD4$^+$ T cells were labeled with CFSE before the coculture with AML cells, and then monitored for the dilution of cell-associated fluorescence by flow cytometry. After coculture with AML cells, no significant proliferation was observed for CD25$^+$ and CD25$^-$ cells, both in medium alone or supplemented with IL-2 (Figure S2). To assess the tendency of T cells to undergo apoptosis, purified CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were incubated with IDO$^+$ AML cells and stained with Annexin-V

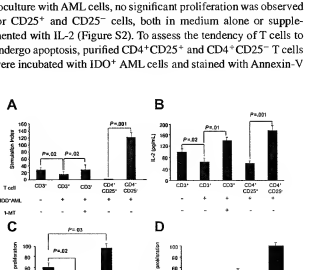
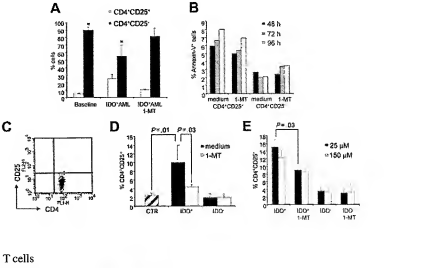


Figure 4. AML-induced CD4$^+$CD25$^+$ T cells are T_{reg} cells. (A-B) Secondary allogeneic MLRs consisting of APCs and total or purified T cells previously cultured with IDO-expressing AML cells in the presence and absence of 1-MT (1000 μM). Naive T cells were used as control sample. APC/T-cell ratio equals 1:10. Proliferation results are expressed as stimulation index. IL-2 concentration was measured in 24-hour coculture supernatants. Data are the mean ± SD of 7 independent experiments. (C-D) Naive CD3$^+$ T cells were stimulated by allogeneic APCs in the presence of total or purified T cells previously cultured with IDO-expressing AML cells, and tastad for proliferation (C). Increasing numbers of purified CD4$^+$CD25$^+$ T cells obtained after culture with IDO-axpressing AML cells were added to MLR cultures (D). Responder and suppressor cells were obtained from the same donor. Experimental T-cell proliferation was compared with that observad in the presence of control T cells and expressed as a parcentage of inhibition. Results are the mean ± SD of 6 indepondent experiments.



and propidium iodide at different time points. $CD4^+CD25^+$ T cells were shown to have increased apoptosis over $CD25^-$ cells, which, however, was not affected by the addition of 1-MT to cultures (Figure 5B). Taken together, these data suggest that the increase of $CD4^+CD25^+$ T cells after culture with IDO^+ AML cells is neither due to active proliferation nor to apoptosis.

To test the conversion hypothesis, highly purified $CD4^+CD25^-$ T cells were used (Figure 5C). Figures 5D and S3 show that the percentage of $CD25^+$ cells increased over that observed after coculture with IDO^- AML cells or with control normal MNCs ($P = .01$). Moreover, the addition of 1-MT to cultures significantly inhibited the increase of $CD25^+$ cells induced by IDO^+ AML cells ($P = .03$), but not by IDO^- AML cells (Figure 5D). Similar results were observed by cultivating T cells in the presence of AML-derived conditioned medium, as previously described (Figure 5E). In contrast to the results observed with total $CD4^+$ T cells (Figure S2), highly purified $CD4^+CD25^-$ T cells, which had been depleted of $CD4^+CD25^+$ T cells (Figure 5C), were induced to proliferate by AML cells, and converting $CD4^+CD25^+$ T cells showed a significant rate of proliferation (Figure S4). Moreover, the effect on proliferation was significantly increased by the addition of IL-2 in $CD4^+C25^+$, but not in $CD4^+CD25^-$ cells. The conversion of $CD4^+CD25^-$ into $CD4^+CD25^+$ T cells was stable, since purified $CD4^+CD25^+$ T cells obtained after culture with IDO^+ AML cells did not revert to $CD25^-$ T cells once IDO^+ cells were removed or 1-MT was added to cultures (Figure S5). However, cultivating T cells and AML cells in a transwell resulted in a significant reduction of the conversion effect, thus suggesting that cell-to-cell contact may be required to induce this phenomenon under such experimental conditions (Figure S6). The different results obtained with AML-derived conditioned medium and those from transwell experiments may be interpreted in light of the different numbers of stimulating AML cells that were used.

These data suggest that IDO expressed by AML cells increases T_{reg} cells by converting $CD4^+CD25^-$ into $CD25^+$ T cells.

Inhibition of IDO prevents tumor-mediated expansion of $CD4^+CD25^+$ T regulatory cells by blocking the conversion of $CD4^+CD25^-$ cells

To further explore the capacity of IDO-expressing tumors in converting $CD4^-CD25^-$ into $CD4^-CD25^+$ T_{reg} cells, we turned to an in vivo murine system. We found that A20 lymphoma/leukemia expresses IDO both at the RNA and protein levels (Figure 6A) as

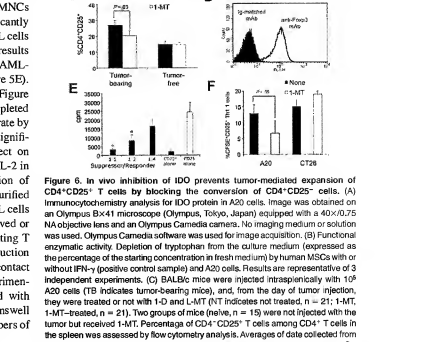


Figure 6. In vivo inhibition of IDO prevents tumor-mediated expansion of $CD4^+CD25^+$ T cells by blocking the conversion of $CD4^+CD25^-$ cells. (A) Immunocytochemistry analysis for IDO protein in A20 cells. Image was obtained on an Olympus BX41 microscope (Olympus, Tokyo, Japan) equipped with a 40×/0.75 NA objective lens and an Olympus Camedia camera. No imaging medium or solution was used. Olympus Camedia software was used for image acquisition. (B) Functional enzymatic activity. Depletion of tryptophan from the culture medium (expressed as the percentage of the starting concentration in fresh medium) by human MSCs with or without IFN-γ (positive control sample) and A20 cells. Results are representative of 3 independent experiments. (C) BALB/c mice were injected intraspinically with 10^6 A20 cells (TB indicates tumor-bearing mice), and, from the day of tumor injection, they were treated or not with 1-D and L-MT (NT indicates not treated, $n = 21$; 1-MT, 1-MT–treated, $n = 21$). Two groups of mice (naïve, $n = 15$) were not injected with the tumor but received 1-MT. Percentage of $CD4^+CD25^+$ T cells among $CD4^+$ T cells in the spleen was assessed by flow cytometry analysis. Averages of data collected from experiments independently performed are reported. The data report the mean ± SD of independent experiments. (D) Intracellular Foxp3 expression in purified $CD4^+CD25^-$ T cells obtained from splenocytes of tumor-bearing mice. Results are representative of 4 independent experiments. (E) Anti-CD3 mediated proliferation of naïve $CD4^+CD25^-$ T cells in the presence of increased numbers of $CD4^+CD25^+$ cells obtained from splenocytes of tumor-bearing mice (■). As a positive control, $CD4^+CD25^-$ cells were stimulated in the absence of $CD4^+CD25^+$ T cells (□). Proliferation was evaluated after 3 days by thymidine incorporation assay. Results are expressed as cpm and represent the mean ± SD of 4 independent experiments. *$P = .03$, experimental versus control sample. (F) In vivo conversion experiments. Purified $Thy1.1^+$ $CD4^+CD25^-$ T cells (10×10^6) were transferred into BALB/c mice bearing A20 or CT26 tumors. After 10 days, spleens were collected and labeled with Thy1.1, CD4, and CD25; $Thy1.1^+$ $CD4^+$ cells were gated and analyzed for CD25 and CFSE expression. Cumulative data of the $CFSE^{lo}CD25^+Thy1.1^+$ cells conversion in spleens cells are reported and represent the mean ± SD of 4 experiments.

well as reduces the concentration of tryptophan from culture medium (Figure 6B). Thus, we assessed whether intrasplenic injection of A20 cells would increase the frequency of $CD4^+CD25^+$ T cells. As shown in Figure 6C, the percentage of $CD4^+CD25^+$ T cells was higher than that of non–tumor-bearing mice. Such increase was time dependent and maximal at day 25 after tumor challenge (data not shown) and, more important, was reduced by the treatment of tumor-bearing mice with 1-MT ($P = .03$). Purified $CD4^+CD25^+$ T cells derived from spleens of tumor-bearing mice were both phenotypically and functionally T_{reg} cells, as shown by Foxp3 expression and the ability to suppress anti-CD3–mediated T-cell proliferation, respectively (Figure 6D-E). To investigate whether conversion may be the mechanism of T_{reg} cell expansion in IDO^+ tumor-bearing mice, $CD4^+CD25^-$ T cells, purified from spleens of Thy 1.1 congenic mice, were labeled with CFSE and inoculated into Thy1.2 mice bearing IDO^- A20 tumors. For control tumor, we used the CT26 colon carcinoma, which lacks IDO expression (data not shown), but has been recently shown to expand a population of well-characterized T_{reg} cells by conversion of $CD4^+CD25^-$ into $CD25^+$ T cells.[25] In both cases a group of mice was treated with I-D and L-MT, as previously described.[6] After about 10 days from tumor challenge, spleens were collected and $Thy1.1^+$ $CD4^+$ donor lymphocytes were analyzed for the expression of CD25 as function of conversion and for CFSE dilution as a function of proliferation. Both A20 and CT26 tumors were capable to induce the conversion of $CD4^+CD25^-$ into $CD4^+CD25^+$ T cells (Figure 6F). However, the treatment with 1-MT was effective in blocking conversion of $CD4^+CD25^-$ into $CD4^+CD25^+$ T cells ($P < .05$) in A20-bearing mice but not in CT26-bearing mice. Moreover, no significant difference was observed as for CFSE dilution (data not shown), indicating that the expansion of T_{reg} cells induced by IDO-expressing tumor cells was due to conversion of $CD25^-$ into $CD25^+$ T cells in the absence of proliferation.

Collectively, these data demonstrate that IDO expression by tumor is directly responsible for in vivo T_{reg} cells expansion by conversion of $CD4^+CD25^-$ into $CD25^+$ $Foxp3^+$ T cells.

Discussion

Since its demonstration as a potent immunosuppressive agent, IDO has been widely investigated for the induction of immunologic tolerance.[26] Recently, IDO has been shown to be expressed in a wide variety of solid tumors and to prevent T-cell–mediated immunity in mouse tumor models.[6] However, little is known about the mechanism(s) by which IDO-expressing tumor cells inhibit antitumor immunity. In this report, we show that IDO, which is constitutively expressed in a significant portion of patients with AML at diagnosis, directly expands $CD4^-CD25^+$ T_{reg} cells by the conversion of $CD4^-CD25^-$ T cells.

Tumor cells, including leukemia cells, are known to create an inhibitory microenvironment for the immune system,[27] which could be counteracted by the optimal secretion of immunomodulatory cytokines, such as IL-12.[28] Recent investigations have established the role of IDO in inducing tolerance to tumors.[6,18] Published data demonstrate that the antitumor effect of IDO blockade was completely dependent on the presence of a fully competent immune system, thus suggesting that IDO acts by deregulating the host immune response. In the present study, we show that in patients with AML IDO expression is associated with an increased number of circulating $CD4^+CD25^+Foxp3^+$ T cells. Such correlation is physiologic in the placenta, where decidua cells expressing IDO are fully infiltrated by $CD4^+CD25^+$ T cells,[15] which have a major role in the induction of maternal tolerance against fetal alloantigen.[16] Accordingly, our data demonstrate that IDO-expressing leukemia cells expand, in vitro and in vivo, a population of $CD4^+CD25^+$ $Foxp3^+$ T cells, which functionally act as bona fide T_{reg} cells.

Different mechanisms have been proposed for IDO-mediated immunoregulation during infection, pregnancy, autoimmunity, transplantation, and neoplasia.[1] Local depletion of tryptophan and/or the production of proapoptotic kynurenines are considered responsible for the multiple effects on lymphocyte proliferation and survival after IDO induction.[1,2,29] In particular, Th1 but not Th2 clones can rapidly undergo cell death in presence of low concentrations of tryptophan metabolites of the kynurenine pathway, such as 3-hydroxyanthranilic and quinolinic acids.[30] In the present study, we were not able to demonstrate any role for IDO in the induction of apoptosis of T-cell subsets. Conversely, we have been able to demonstrate, in vitro, that IDO-expressing AML cells directly increase T_{reg} cells through a mechanism of conversion from $CD4^+CD25^-$ T cells. Moreover, although mice experiments cannot definitely rule out the possibility that T_{reg} cell frequencies may be affected in vivo by several different mechanisms, our results suggest that conversion may be an important pathway by which IDO-expressing tumors expand T_{reg} cells.

Originally thought to be of restricted thymic origin, recent evidence indicates that T_{reg} cells can also be generated in the periphery upon subimmunogenic stimuli (ie, in the presence of suboptimal doses of antigen and APC activation),[31,32] a situation resembling tumor-host interaction. Accordingly, murine tumors of different histology are capable to expand T_{reg} cells by converting $CD4^+CD25^-$ into $CD25^+$ T cells.[25] Colombo et al[25] postulated that tumor-derived soluble factors, such as transforming growth factor (TGF)–β1,[33,34] which are known to regulate the conversion of $CD4^+CD25^-$ into $CD4^+CD25^+$ T cells,[35] may play a critical role in inducing the conversion into T_{reg} cells. Although we could not rule out that, in our system, a fraction of converting T cells may be generated in the presence of leukemia-derived production of soluble factors, such as TGF-β1, we found that T-cell conversion was completely abrogated by IDO blockade in AML cells. These data point to a direct role of IDO production in the expansion of converting T_{reg} cells by AML cells and are in agreement with the results by Puccetti et al demonstrating that in a nontumoral mouse model, tryptophan catabolism favors the emergence of $CD25^+Foxp3^+$ T_{reg} cells by conversion from $CD25^-Foxp3^-$ cells.[36] It remains to be elucidated how the modulation of tryptophan catabolism by IDO-expressing AML cells may be implicated in the conversion of $CD4^+CD25^-$ cells into $CD4^+CD25^+$ cells. In particular, the accumulation of small molecules within tumor microenvironment has been recently demonstrated to affect significantly tumor-infiltrating cell populations.[37] Here we show that the conditioned medium obtained from IDO^+ but not IDO^- AML cells was capable, per se, to induce the conversion of $CD4^+CD25^-$ T cells. These data suggest that IDO^+ AML cells induce a tumor microenvironment containing reduced concentrations of tryptophan and high concentrations of kynurenine, which may have a role in AML-induced expansion of T_{reg} cells by conversion of $CD4^+CD25^-$ T cells.

In conclusion, IDO production by AML cells directly increase T_{reg} cells through the conversion of $CD25^-$ into $CD25^+$ T cells. IDO expression can be regarded as a novel mechanism of leukemia escape from immune control and its inhibition may represent a novel antileukemia therapeutic strategy.

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Authorship

Contribution: A.C. was responsible for design of the research, flow cytometry, human functional tests, statistical analyses, writing of the manuscript, and review of the accuracy of the reported results; G.B., S.R., S.P., A.I., E.F., and V.S., for flow cytometry, functional tests, immunocytochemistry, and ELISAs; M.A., for molecular biology; B.V., for mouse experiments; I.D. and F.F., for HPLC analysis; M.M., A.L.H., and M.B., for critical review of the manuscript; M.P.C., for contribution to manuscript writing; and R.M.L., for contribution to the research plan and manuscript writing.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Exhibit C

6 Dik WA, Brahim W, Braun C, Asnafi V, Dastugue N, Bernard OA *et al.* CALM-AF10+ T-ALL expression profiles are characterized by overexpression of HOXA and BMI1 oncogenes. *Leukemia* 2005; **19**: 1948–1957.

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Acute myeloid leukemia cells constitutively express the immunoregulatory enzyme indoleamine 2,3-dioxygenase

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Indoleamine 2,3-dioxygenase (IDO) is a key enzyme in the tryptophan metabolism, which catalyzes the initial rate-limiting step of tryptophan degradation along the kynurenine pathway.[1] Tryptophan starvation by IDO consumption inhibits T-cell activation,[1,2] whereas products of tryptophan catabolism, such as kynurenine derivatives and O_2 free radicals, regulate T-cell proliferation and survival.[1,3] Thus, IDO has a profound

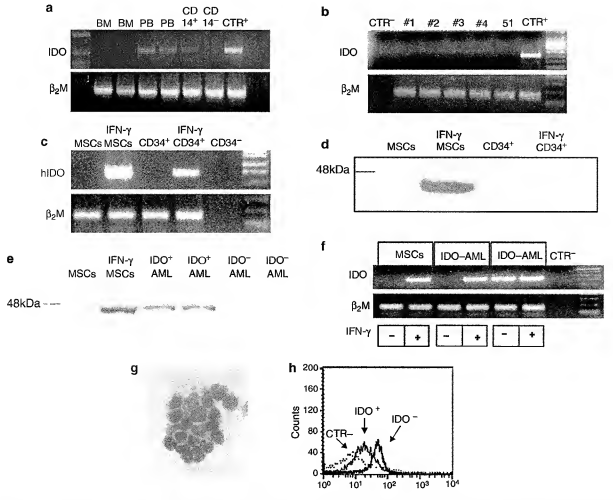

Figure 1 IDO expression by normal and leukemic hematopoietic cells. (a) *INDO* mRNA expression by normal BM- and PB-derived MNCs ($n=2$) and by PB $CD14^+$ and $CD14^-$ cells ($n=1$). IFN-γ-stimulated MSCs were used as positive control sample (CTR^+). Data are representative of four independent experiments. (b) *INDO* mRNA expression by highly purified $CD34^+$ cells obtained from the BM of five healthy donors. IFN-γ-stimulated MSCs were used as positive control sample (CTR^+). Data represent five independent experiments. (c) *INDO* mRNA expression by $CD34^+$ cells with or without IFN-γ. IFN-γ-stimulated MSCs were used as positive control sample. Results are representative of four independent experiments. (d) Western blot analysis for IDO protein was performed in MSCs and $CD34^+$ cells with or without IFN-γ stimulation. Results are representative of three independent experiments. (e) Western blot analysis for IDO protein was performed in IDO^+ and IDO^- AML samples. For each group, two representative samples are reported. MSCs with or without IFN-γ stimulation were used as negative and positive controls, respectively. (f) *INDO* mRNA expression by MSCs, IDO^- and IDO^+ AML cells with or without IFN-γ. Results are representative of four independent experiments. (g) IDO protein in IDO^- AML cells stimulated with IFN-γ. Results are representative of four independent experiments. (h) Flow cytometry for IFN-γ receptor on IDO^- and IDO^+ AML cells. Control isotype-matched monoclonal antibody was used.

immunosuppressive activity. Accordingly, cell populations expressing IDO, including regulatory dendritic cells (DCs) and bone marrow (BM)-derived mesenchymal stem cells (MSCs), have the capacity to suppress T-cell responses to auto- and allo-antigens.[4,5] In these cells, the expression of IDO gene and protein is critically regulated by interferon (IFN)-γ.[4,5]

Recent evidence has demonstrated that a wide variety of human solid tumors express the functionally active IDO protein, which acts as a mechanism of escape from the immune response.[6] In particular, transfecting IDO into tumor cells prevents their rejection by pre-immunized hosts and the antitumor effect of IDO blockade is completely dependent on the presence of a fully competent immune system, thus suggesting that IDO acts by deregulating the host immune response. Despite the extensive work on solid tumor cells, no data are currently available for IDO expression and T-cell inhibitory function in human leukemic cells and normal hematopoietic stem cells (HSCs).

To test whether BM-derived hematopoietic cells express IDO, we firstly analyzed total BM-derived mononuclear cells (MNCs) obtained from healthy volunteers. As shown in Figure 1a, BM-derived MNCs were negative for *INDO* expression, whereas *INDO* was detected in $CD14^+$ monocytes purified from the peripheral blood (PB). Then, we analyzed highly purified $CD34^+$ HSCs from BM of healthy donors. Figure 1b shows that 5/5 $CD34^+$ cell samples did not express *INDO*. We then asked whether $CD34^+$ cells could express IDO gene and protein upon exposure to optimal concentration of IFN-γ. $CD34^+$ HSCs from healthy donors expressed *INDO* after incubation with IFN-γ (Figure 1c), but were negative for IDO protein, as evaluated by both immunocytochemistry (data not shown) and Western blot analyses (Figure 1d). Conversely, BM non-hematopoietic IFN-γ-stimulated MSCs expressed IDO mRNA (Figure 1c) and protein (Figure 1d). These data suggest that BM-derived hematopoietic and non-hematopoietic cells follow different pathways for IDO expression, perhaps owing to a different susceptibility to IFN-γ modulation. In addition, normal IFN-γ-stimulated $CD34^+$ cells express IDO mRNA, but not IDO protein, thus indicating a post-transcriptional regulation of IDO protein expression in normal HSCs.

To test whether leukemic cells express IDO, primary AML cells collected from 76 AML patients at diagnosis (Supplementary Table 1) were tested for IDO expression. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis demonstrated that 40/76 (52%) AML samples were positive for *INDO* mRNA. Accordingly, IDO protein was expressed only in RT-PCR-positive AML samples as evaluated both by immunocytochemistry (data not shown) and Western blot analyses

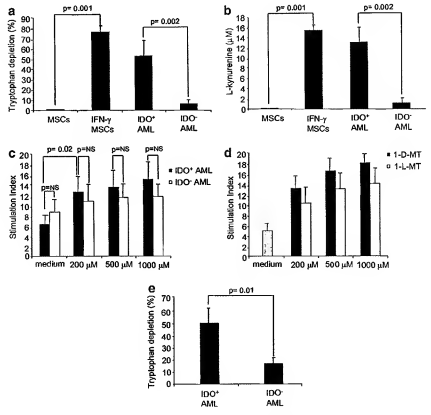

Figure 2 IDO functional activity. Functional enzymatic activity is shown in (a) as depletion of tryptophan (expressed as the percentage of the starting concentration in fresh medium) and in (b) as L-kynurenine production by MSCs with or without IFN-γ and AML cells (10^6 cells/well). Supernatants were analyzed after 72 h of culture. Results are representative of four independent experiments. (c) Allogeneic MLRs using IDO^+ and IDO^+ AML cells as stimulators and purified $CD3^+$ T cells as responders in the presence and absence of different concentrations of 1-D-MT. APC/T-cell ratio = 1/10. Results are expressed as stimulation index and report the mean ± s.d. of 10 independent experiments. (d) Allogeneic MLRs using IDO^+ AML cells as stimulators and purified $CD3^+$ T cells as responders in the presence and absence of different concentrations of 1-D-MT and 1-L-MT. APC/T cell ratio = 1/10. Results are expressed as stimulation index and report the mean ± s.d. of three independent experiments. (e) Depletion of tryptophan (expressed as the percentage of the starting concentration in fresh medium) from day 5 MLR cultures containing IDO^- and IDO^+ AML cells as stimulators. Results report the mean ± s.d. of five independent experiments.

(Figure 1e). Similar to normal CD34$^+$ cells, IDO$^-$ AML cells expressed *INDO* mRNA, but not IDO protein when exposed to optimal concentrations of IFN-γ (Figure 1f and g). IFN-γ stimulation of IDO$^+$ AML cells did not upregulate *INDO* mRNA (Figure 1f). Interestingly, the expression of IFN-γ receptor (CD119) was significantly downregulated in IDO$^+$ as compared to IDO$^-$ AML cells (Figure 1h). The median fluorescence intensity (MFI) of IDO$^-$ and IDO$^+$ AML cells was 87±10 and 32±14, respectively ($P=0.01$). Taken together, these data demonstrate that, similar to solid tumors,[6] a significant portion of AML samples shows a constitutive expression of IDO. Similarly to normal HSCs, a subset of AML samples lacks IDO expression, which can be induced after exposure to IFN-γ only at molecular, but not at protein level. Thus, the differential expression of IDO among normal CD34$^+$ HSCs and leukemic cells supports the hypothesis that, in a subset of AML patients, the constitutive expression of IDO protein is associated with the leukemic transformation and may play a role in leukemia development.

A further set of experiments was performed to test the functional activity of IDO protein expressed by AML cells. IDO$^+$ and IDO$^-$ AML cells were cultured for 48 h and then supernatants were analyzed for L-tryptophan and L-kynurenine concentrations. In these experiments, IFN-γ-stimulated MSCs were used as positive control. As shown in Figure 2a and b, IDO from AML cells was functionally active, resulting in the reduction of tryptophan and the increase of L-kynurenine in the culture medium. Accordingly, IDO$^-$ AML cells showed a very little capacity of tryptophan depletion from culture medium and, more importantly, they were not capable to produce kynurenine metabolite. To test whether IDO$^+$ and IDO$^-$ AML cells were different in their ability to stimulate T-cell proliferation, AML cells were used as stimulators for allogeneic CD3$^+$ T cells in a standard mixed lymphocyte reaction (MLR). As shown in Figure 2c, T-cell proliferation induced by IDO$^-$ AML cells was slightly, albeit not significantly, increased over that observed with IDO$^+$ AML cells. However, in agreement with our data on IDO expression, the addition of increasing concentrations of 1-D-MT resulted in a significant increase of T-cell proliferation only when IDO$^+$, but not IDO$^-$ AML cells were used as stimulators ($P=0.02$). Similar results were observed when the L isomer of 1-MT, which is considered a better IDO-inhibitor than 1-D-MT, was used in MLR cultures (Figure 2d). Moreover, tryptophan depletion from day 5 MLR supernatants was significantly increased in presence of IDO$^+$ AML cells as compared to IDO$^-$ AML cells (Figure 2e).

Frequencies of *INDO* gene expression among French-American-British (FAB) subgroups were analyzed. There was a higher, but not statistically significant percentage of AML samples of M5 FAB subtype in IDO$^+$ versus IDO$^-$ patients ($P=0.06$) (Supplementary Figure 1) and this finding may be consistent with the constitutive expression of *INDO* gene in hematopoietic cells of monocytic origin. There was no significant difference with regard to sex distribution among IDO$^+$ and IDO$^-$ groups (data not shown). IDO$^+$ and IDO$^-$ AML patients were comparable for the most relevant prognostic parameters at diagnosis such as age, circulating blast cell count, percentage of high-risk cytogenetic alterations and secondary disease (Supplementary Table 2). Notably, most IDO$^+$ AML samples had a normal karyotype and lacked leukemia-associated molecular markers (59%).

In conclusion, this is the first report demonstrating that the functionally active form of IDO protein is constitutively expressed in a significant portion of AML cells, whereas it is not detectable in normal hematopoietic BM cells, including CD34$^+$ HSCs. Given the role of IDO as immunosuppressive agent, the finding of IDO activity in AML cells may be considered a novel pathway of leukemia escape from immune response. Further studies are ongoing to better elucidate the mechanism(s) IDO-expressing AML cells exert to impair anti-leukemia immunity.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Exhibit D

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Leukemia Research
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Brief communication

Indoleamine 2,3-dioxygenase activity of acute myeloid leukemia cells can be measured from patients' sera by HPLC and is inducible by IFN-γ

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Abstract

The enzyme indoleamine 2,3-dioxygenase (IDO) converts tryptophan to kynurenine, blocking T-cell activation and inducing immunosuppression. In patients with acute myeloid leukemia (AML), the serum kynurenine/tryptophan ratio (Kyn/Trp) was raised, suggesting a higher IDO activity than in healthy people. Patients with higher Kyn/Trp ratios showed lower survival. IDO activity was also detected in AML cells after exposure to IFN-γ *in vitro*, suggesting that the higher Kyn/Trp ratio in serum of AML patients might have resulted from stimulated leukemic blast cells. Thus, in AML, the activity of IDO can be easily monitored, providing a tool for future clinical testing of IDO-blocking drugs.

Keywords: IDO; AML; Kynurenine; Tryptophan; IFN-γ

1. Introduction

Indoleamine 2,3-dioxygenase (IDO) degrades the essential amino-acid tryptophan and produces kynurenine that is converted in several metabolites through downstream enzymes. IDO induces peripheral immune tolerance and immunosuppression by reducing the local concentration of tryptophan [1]. Kynurenine and its derivatives also inhibit T- and NK-cells. IDO activity can be induced in antigen-presenting cells, by reverse signalling, through B7.1 and B7.2 binding by CTLA4 expressed on T-regulatory (Treg) cells. IDO can also be expressed by several types of cells at inflammation sites, and may prevent excessive local tissue damage. IDO is also expressed in cancer cells or in lymph nodes that drain tumours, and it contributes to the immuno-escape of tumour cells [1]. Tumour cells express IDO after exposure to inflammatory factors like INF-γ or toll-like-receptor (TLR) ligands. Moreover, treatment of tumour-bearing mice with IDO inhibitors can make cancer vaccines more effective. Thus, in future, specific IDO inhibitors might be included in treatments involving cancer vaccines and chemotherapy.

Recently, several groups have reported that in acute myeloid leukemia (AML), blast cells express IDO [2,3], and this can convert CD25− T cells into CD25+ Treg T cells [3]. Thus IDO seems an interesting target for future immunotherapy strategies in AML. However, levels of IDO mRNA or protein may not necessarily reflect levels of IDO activity. The enzyme is tightly regulated, and measuring actual enzyme activity is necessary for evaluating its role in AML, and for

Abbreviations: Kyn, kynurenine; Trp, tryptophan; IDO, indoleamine 2,3-dioxygenase.

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future clinical trials with IDO inhibitors. We assessed IDO activity in a cohort of AML patients by measuring kynurenine and tryptophan in the patients' sera, and in the supernatant of AML cells exposed to various inflammatory signals. We show here that IDO activity is detectable in many AML patients, and that AML cells can produce kynurenine after stimulation by INF-γ.

2. Patients and methods

Blast cells and sera were isolated from 184 adult patients after informed consent was gained in accordance with the Helsinki Declaration. Study was approved by the Institutional Review Board of Tumorotheque/CHU Lille. IDO mRNA was quantified by RQ-PCR. Data were normalized by levels of Abl RNA (Abl: Applied Biosystems, Foster City, CA). Primers and probes were designed with the Primer Express software and purchased from Applied Biosystems. Primers: F-hIDO 5′-GGTCATGGAGATGTCCGTAA-3′; R-hIDO 5′-ACCAATAGAGAGACCAGGAAGAA-3′; probe: 6-FAM-ATGCATCACCATGGCA-MGB. RQ-PCR was carried out with the TaqMan Universal Master Mix on an ABI PRISM 7700HT sequence detection system (Applied Biosystems).

IDO protein was detected by Western blot with mouse anti-IDO mAb (Chemicon, Temecula, CA). Tryptophan and kynurenine in sera were quantified by HPLC (Waters LC-600 with two detectors) as previously described [4]. Kynurenine in the supernatant of THP-1 cell line and AML blast cells were quantified spectrophotometrically as previously described [5].

Statistics were performed using the SPSS 13.0 software (SPSS, Chicago, IL).

3. Results and discussion

We first measured IDO mRNA in bone marrow samples collected at diagnosis from 184 AML patients, and compared them to the THP-1 leukemic cell line, either unstimulated, or stimulated with IFN-γ (Fig. 1A). THP-1 cells express a high level of IDO and were used as a positive control in T-cell inhibition experiments. IDO mRNA was detected in most AML samples. Expression levels varied, but were within the range observed for THP-1 (stimulated or unstimulated). No correlation could be found with FAB subtype, white blood cell count, or karyotype. Recently, Curti et al. also analyzed by RT-PCR bone marrow cells from healthy donors and patients with AML. They found IDO mRNA in 52% of AML samples but in none of the control samples [2]. Interestingly, they reported some AML samples expressing IDO mRNA but not IDO protein. IDO protein needs posttranslational modifications to gain full activity [1]. Thus in AML, IDO mRNA levels may not indicate IDO activity accurately.

We then evaluated IDO activity by measuring tryptophan and kynurenine in patients' sera at diagnosis. Kynurenine levels were significantly higher, and tryptophan levels were significantly lower than in healthy controls (Fig. 1B and C). Kynurenine/tryptophan ratios were higher than in our healthy controls (median 0.046 vs. 0.029), and also higher than previously reported in non-malignant disease and other healthy controls (Fig. 1D) [4,6]. We did not find any correlation between IDO mRNA level in AML cells, and serum levels of kynurenine, tryptophan, or the kynurenine/tryptophan ratio. HPLC results were not correlated with age, sex, leukocytosis, FAB, karyotype, FLT-3 or NPM mutations.

We then evaluated the possible impact of IDO on survival. Overall survival was not correlated with IDO mRNA level. However, when patients were divided into two groups, survival of patients with serum kynurenine/tryptophan >0.075 was significantly lower than in the rest (Fig. 1E). These data suggest that levels of kynurenine and tryptophan in patients' sera may reflect an increased IDO activity in AML blasts, which might affect outcome.

In tumour cells, the signals that induce IDO activity remained to be identified. Even in normal cells, the mechanism of IDO regulation is largely unknown. Curti et al. showed that AML cells positive for IDO mRNA also expressed the protein [2]. In AML cells originally lacking mRNA, IFN-γ induced the mRNA, but not the protein. The same results were observed in CD34+ cells from a healthy volunteer, suggesting that some AML patients might express IDO constitutively. In dendritic cells, full induction of IDO activity requires two signals. For instance, PGE2 induces IDO mRNA transcription, and a second signal, through TNFα receptor or TLR, is necessary to activate the enzyme [5].

We first studied inducers of IDO activity on THP-1 cell line, by measuring kynurenine in supernatants. Induction of IDO activity required both IFN-γ (500 IU/ml) and LPS (2.5 μg/ml). IFN-γ alone induced IDO activity at higher doses (>750 IU/ml; Fig. 2A and B). Thus, IDO can be activated in THP-1 by similar stimuli to those used by normal dendritic cells.

Using the same conditions, we then tested 15 AML samples with >99% blast cells for kynurenine production (Fig. 2C). Basal production was low (median 0.50 μmol/l, range 0–13.7). IFN-γ alone (500 IU/ml) induced kynurenine production (median 11.6 μmol/l, range 0.04–33.4), but 250 IU/ml TNF-α, 5 μM PGE2, or 2.5 μg/ml LPS alone did not. Combining IFN-γ, LPS, and TNF-α induced kynurenine production (median 17.6 μmol/l, range 6.41–33.8). Specificity was verified with pyrrolidine dithiocarbamate (PDTC), an antioxidant and inhibitor of IDO activity at the post-translational level through limiting the availability of heme for formation of IDO holoenzyme. PDTC almost stopped kynurenine production. IFN-γ induced increased IDO protein level which was not affected by PDTC, confirming the posttranslational effect of this compound (Fig. 2D). Thus, contrary to normal dendritic cells and THP-1 cells, IDO activity in AML cells requires only IFN-γ. These results contrast partially with those of Curti et al. who reported substantial kynurenine production from unstimulated cells expressing IDO protein [3]; however, they studied only three samples. In our experiments, only one sample of the 15 produced a substantial level of kynurenine without stimulation. Since most of our AML patients showed high Kyn/Trp levels in

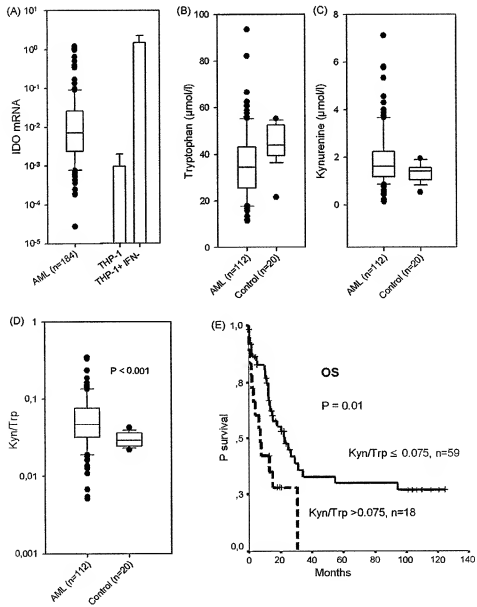

Fig. 1. Analysis of IDO mRNA and IDO activity in patients with AML. (A) RQ-PCR analysis of IDO mRNA in 184 patients with AML, and in the THP-1 leukemic cell line treated with 500 IU/ml IFN-γ for 24 h. Levels are relative to the Abl housekeeping gene. (B) Serum tryptophan levels measured by HPLC in sera of 112 patients with AML, or in 20 controls. (C) Serum Kynurenine levels in the same subjects as (B). (D) Serum kynurenine/tryptophan ratio (Kyn/Trp) in the same subjects as (B). Groups were compared using Student's *t*-test. (E) Overall survival (OS) of AML patients according to their serum Kyn/Trp ratio. OS was estimated according to the Kaplan–Meier method and groups were compared using the log-rank test.

sera, IDO activity *in vivo* likely results from blast stimulation in some patients, and from constitutive activity in others. The high Kyn/Trp ratio might also result from mesenchymal stem cells or dendritic cells activated indirectly by leukemic cells: in some solid tumours, IDO activity is detected both in tumour cells, and tumour-draining lymph-nodes. We therefore hypothesize that *in vivo*, IFN-γ comes from autologous T-cells, which recognise tumour antigens, but there may be other stimuli also. For instance, bacterial infections may provide LPS that acts directly through toll-like receptors, or more likely indirectly by inducing TNFα and IFN-γ.

In summary, in patients with AML, IDO activity can be monitored by measuring its metabolites in sera, and appears correlated with outcome. The results probably reflect IDO activity in AML cells activated by IFN-γ and/or other stimuli. Several animal models have demonstrated a therapeutic effect of IDO inhibitors, like 1-methy-tryptophan, which enhanced cancer vaccine effects [7]. Synergy between IDO inhibitors

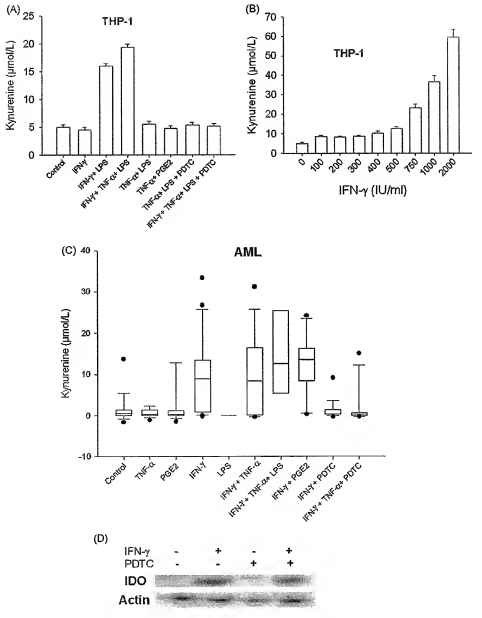

Fig. 2. Kynurenine production by leukemic cells. (A) Kynurenine production measured by spectrophotometric assay in supernatant from THP-1 leukemic cells, incubated for 24 h with 500 IU/ml IFN-γ, 2,5 µg/ml LPS, 5 µM PGE2, or 250 IU/ml TNFα alone or in combination. PDTC (125 µM) was added to block IDO activity. (B) Kynurenine production from THP-1 incubated with various concentrations of IFN-γ and assessed as in (A). All experiments were made two times in triplicate. Data represent mean and S.D. (C) Kynurenine production from 15 AML samples, incubated with various cytokines and 125 µM PDTC, and assessed as in (A). (D) Western blot showing IDO protein expression in one AML sample exposed for 24 h to 500 IU/ml IFN-γ, or to the IDO inhibitor PDTC alone, or in combination.

and chemotherapy has also been reported [8]. IDO inhibitors might help the immune system to clear residual leukemic cells after chemotherapy. Monitoring IDO activity provides a useful tool for future clinical trials of IDO inhibitors in AML.

Conflicts of Interest

No financial competing interests.

Acknowledgments

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Contributions. BQ designed the study; SC, CB, MI, ML, CD, IB, VB performed the research and analyzed the data; SC and BQ wrote the paper.

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Exhibit E

High *INDO* (indoleamine 2,3-dioxygenase) mRNA level in blasts of acute myeloid leukemic patients predicts poor clinical outcome

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ABSTRACT

Indoleamine 2,3-dioxygenase degrades the amino acid tryptophan which is essential for T cells. Tryptophan depletion causes T-cell cycle arrest and solid tumors that express high levels of indoleamine 2,3-dioxygenase can create immune suppression. Recently, blasts of patients with acute myeloid leukemia were shown to express indoleamine 2,3-dioxygenase. We determined *INDO* (encoding gene for indoleamine 2,3-dioxygenase) mRNA expression in leukemic blasts of 286 patients with acute myeloid leukemia by gene-expression profiling. Results were validated by quantitative polymerase chain reaction analysis in blasts of an independent cohort of 71 patients. High *INDO* expression was correlated to significantly shortened overall and relapse-free survival. Correlation of *INDO* expression to relevant known prognostic factors and survival identified high *INDO* expression as a strong negative independent predicting variable for overall and relapse-free survival. Inhibition of indoleamine 2,3-dioxygenase expressed by myeloid leukemic blasts may result in breaking immune tolerance and offers new therapeutic options for patients with acute myeloid leukemia.

Key words: indoleamine 2,3-dioxygenase, *INDO*, acute myeloid leukemia, immunesurveillance, immune-therapy.

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Introduction

Successful immunotherapy requires not only the development of effector T cells, but also the break-through of the local state of immune tolerance that tumors can create. Besides several cellular and soluble factors (like the presence of regulatory T cells and transforming growth factor β),[1] overexpression of the enzyme indoleamine 2,3-dioxygenase can induce tolerance. Indoleamine 2,3-dioxygenase degrades the essential amino acid tryptophan into kynurenine. High levels of indoleamine 2,3-dioxygenase result in immune suppression because T cells undergo cell cycle arrest in G1 phase at low tryptophan levels.[2] Moreover, kynurenine and its derivates are directly toxic for T cells.[3] Biologically relevant indoleamine 2,3-dioxygenase to limit T-cell activation[4] is expressed by interferon-α stimulated antigen presenting cells, in lower intestinal epithelial cells in which non-pathogenic bacteria are frequently present, and in trophoblast cells in which it protects the fetus from attack by maternal cytotoxic T cells.[5]

Tumor induced overexpression of indoleamine 2,3-dioxygenase causes immune-suppression at two levels. First, inhibition of effective T-cell *priming* by antigen presenting cell derived indoleamine 2,3-dioxygenase has been demonstrated in tumor-draining lymph nodes.[6] Second, the effector phase of an anti-tumor immune response is hampered because many human solid tumors themselves express indoleamine 2,3-dioxygenase.[7-9]

In acute myeloid leukemia, acquired mutations of the hematopoietic stem cells block differentiation. The result is accumulation of immature cells in the bone marrow and peripheral blood, often accompanied by suppression of the normal blood cells. With chemotherapy and stem cell transplantation about 70% of patients achieve complete remission but approximately half of these patients relapse from the status of minimal residual disease.[10] The immune system as surveillant in acute myeloid leukemia is unlikely to play a role at the moment of diagnosis (when an enormous tumor burden exists) but this is more likely during the period of minimal residual disease (when the patient has achieved morphological complete remission). We hypothesize that, in this phase of the disease, overexpression of indoleamine 2,3-dioxygenase by myeloid leukemic blasts could hamper immunesurveillance resulting in immune escape and shortened relapse-free and overall survival. With regard to hematologic malignancies, Curti *et al.* reported active indoleamine 2,3-dioxygenase protein in 52% of acute myeloid leukemic samples.[11] They

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also clearly demonstrated that indoleamine 2,3-dioxygenase expressed by human myeloid leukemic cells induces the expansion of $CD4^+CD25^+FOXP3^+$ regulatory T cells in mice.[12] These data presume an important role for indoleamine 2,3-dioxygenase in immune escape. However, negative influence of high indoleamine 2,3-dioxygenase expression on overall survival of patients has only been demonstrated for endometrial and colorectal cancer.[8,9] In the present study we investigated whether the expression of *INDO* (the gene encoding for indoleamine 2,3-dioxygenase) correlates to clinical outcome of patients with acute myeloid leukemia. We determined *INDO* expression in myeloid leukemic blasts of 285 patients by gene-expression analyses and in 71 patients by real-time quantitative polymerase chain reaction (qPCR) and correlated these data to relevant known prognostic factors and survival.

Design and Methods

Patients' characteristics

After informed consent and according to the recommendations as defined in the declaration of Helsinki in 1989, bone marrow derived samples were collected from patients with primary acute myeloid leukemia. Patients were classified according to the French-American-British (FAB) classification.[13] Clinical characteristics of the patients that were selected for gene-expression profiling analysis have been previously described[14] and shown in Table 1. For patients analyzed by qPCR, clinical characteristics are comparable to the clinical characteristics of patients that were analyzed by gene-expression profiling (Table 1).

Cytogenetic risk group was defined as favorable (t(8;21), inv(16) or t(15;17)), standard (neither favorable nor adverse) or adverse (complex karyotype, -5, -7, del(5q), del(7q), abn (3q), t(6;9) or abn (11q23)).

Patients of both groups received therapy according to HOVON (Dutch-Belgian Hematology-Oncology Cooperative Group) protocols. Most patients received 2 cycles of chemotherapy (containing cytarabin combined with idarubicin or amsacrine) followed by an autologous stem cell transplantation or a third cycle of chemotherapy (mitoxantrone and etoposide). Only 3 patients received allogeneic stem cell transplantation. Protocols are available at *www.hovon.nl*. Overall survival was defined as the time period from inclusion to death or last date of follow-up; relapse-free survival was defined as the time period from achievement of complete remission to relapse.

Sample characteristics

Blood and bone marrow derived mononuclear cell fractions from patients with primary acute myeloid leukemia were obtained by Ficoll centrifugation (purity of blasts >80%), cryopreserved and used for gene-expression profiling. For qPCR analysis, $7AAD^-$/AnnexinV$^+$/$CD45^{dim}$ cells from patients with primary acute myeloid leukemia were FACS sorted after thawing (purity of blasts >95%). For comparison, bone marrow from 5 healthy donors was withdrawn and $7AAD^-$/AnnexinV$^-$/$CD45^{dim}$ normal blasts (purity >95%) were FACS sorted.

Table 1. Comparison of clinical characteristics of patients with acute myeloid leukemia whose blasts were analyzed for INDO expression by gene-expression profiling and by qPCR.

Type of analysis	*Gene-expression profiling*	*qPCR*
Number	286	71
Median age in years at diagnosis (range)	44 (15-78)	54 (16-75)
Median white blood cell count at diagnosis (10^9/L, range)	28 (0.3-582)	42 (0.4-282)
Complete remission rate, number (%)	203 (79.6)	55 (78)
Median overall survival in months (range)	13 (0-166)	14.4 (0.03-174)
Median relapse free survival in months (range)	11 (0-166)	16.6 (0.23-173)
FAB classification, number (%)		
AML M0	6 (2)	4 (6)
AML M1	63 (22)	9 (13)
AML M2	66 (23)	20 (19)
AML M3	19 (7)	3 (4)
AML M4	53 (19)	12 (17)
AML M5	65 (23)	20 (28)
AML M6	3 (1)	3 (4)
not determined	10 (3)	
Cytogenetic risk group, number (%)		
Favorable	57 (20)	4 (6)
Standard	176 (62)	40 (56)
Adverse	39 (14)	6(9)
No metaphasis	13 (4)	6 (9)
Not done		15 (21)
FLT3 status, number (%)		
FLT3 ITD pos	78 (27)	19 (27)
FLT3 ITD neg	207 (73)	52 (73)
FLT3 TKD pos	33 (12)	5 (7)
FLT3 TKD neg	252 (88)	66 (93)

Gene-expression profiling and real-time qPCR

Gene-expression levels for *INDO* were determined by using Affymetrix U133A GeneChips[14] (Affymetrix-id for *INDO* 210029_at, M34455 NCBI). For qPCR, RNA was isolated using RNA-Bee solution (Tel-Test Inc, Friendswood, TX, USA). Total RNA was stored at -80°C. cDNA synthesis was performed according to a standardized Europe Against Cancer protocol.[15] PCR amplification was performed with a LightCycler real-time PCR machine (Roche Diagnostics, Almere, the Netherlands). Reaction volumes were 20 µL, consisting of 2 µL cDNA, 2 µL of LightCycler Fast Start DNA SYBR Green Mastermix (Roche) and 0.5 µM reverse and forward primers. $MgCl_2$ was added to a final concentration of 3.5 µM (for *GUS* 4 µM). qPCR conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 45 cycles, each for 15 sec at 95°C, 10 sec at 58°C (for *GUS* 59°C) and 10 sec at 72°C. Relative quantitation of gene-expression was determined using the LB1610 cell line (*see Acknowledgments*). All results were normalized with respect to the β-glucuronidase (*GUS*)

control. Primer sequences were: *INDO* forward: 5'-GTGTTTCACCAAATCCACGA-3', reverse: 5'-CTG-ATAGCTGGGGGTTGC-3', *GUS* forward: 5'-GAA-AATATGTGGTTGGAGAGCTCATT-3', reverse: 5'-CCGAGTGAAGATCCCCTTTTTA-3 (Biolegio BV, (Nijmegen, the Netherlands).

Western blot

For Western Blot analysis, human peripheral blood derived monocytes selected by magnetic separation (positive selection with CD14 microbeads, Miltenyi Biotec, Bergisch Gladbach, Germany) were used. Purity was greater than 97%. Monocytes were incubated for 18 hours with interferon-α. Snap frozen cells were prepared by resuspending in lysis buffer (50 mM Tris-HCl (pH 7.6), 5 mM dithiotreitol, 20 µL PIC (Protease Inhibitor Cocktail; 1 tablet/mL H_2O), 20% glycerol and 0.5% NP-40). Protein content of the supernatant was determined by the Bio-Rad protein assay. Twenty micrograms of total cell lysates were fractionated on a 12.5% polyacrylamide gel containing SDS and transferred onto a PVDF membrane. Indoleamine 2,3-dioxygenase was visualized by luminescence (ECL Kit, Amersham) using Hyperfilm ECL (Amersham) after incubation with anti-human indoleamine 2,3-dioxygenase antibody (clone 10.1, Chemicon, 1:1000) and horseradish peroxidase–coupled rabbit anti-mouse IgG. α-tubulin was detected by incubation with a mouse anti-human α-tubulin monoclonal antibody (Sc-5286 Santa Cruz) by luminescence as above.

Statistical analysis

Statistical analyses were conducted using a SPSS 12.0.1 software program. To determine associations between variables, Spearman's correlation coefficient was used. Differences between patients' characteristics were analyzed with the Mann-Whitney U test. For survival data, Kaplan-Meier curves were compared by means of the log-rank test. To explore the simultaneous effect of several variables on overall and relapse-free survival the Cox regression model was used.

Results and Discussion

Two hundred and eighty-six patients were analyzed for the expression of *INDO* by gene-expression profiling. *INDO* mRNA expression was variable and not significantly associated with any one of the 16 subtypes of acute myeloid leukemia, which were identified on their specific gene-expression signatures,[14] presuming independency of these prognostic clusters (Figure 1A). Moreover, no significant differences were found between INDO mRNA expression of different FAB subtypes and cytogenetic risk profiles. Correlation of *INDO* expression to survival data of 262 patients revealed that all patients with prolonged overall survival had low *INDO* expression. We decided to validate these data by qPCR. Correlation between microarray and qPCR is generally strong, although there is non-concordance in 13-16% of genes.[15] *INDO* mRNA expression levels relative to *GUS* by qPCR nicely correlated to protein levels as determined by Western blot on human peripheral blood interferon-α stimulated monocytes (Figure 1B). We then determined *INDO* mRNA expression levels in 71 patients and 5 healthy donors. *INDO* expression relative to *GUS* in blasts of healthy donors was low (mean 0.538, median 0.32, range 0.06-1.18). *INDO* expression relative to *GUS* in blasts of patients with acute myeloid leukemia was highly variable, but mean and median expression was higher (mean 5.68, median 0.51, range 0-304) than *INDO* expression in blasts of healthy donors.

As in the patient group analyzed by gene-expression profiling, no significant differences in *INDO* expression analyzed by qPCR were found between FAB subtypes and cytogenetic risk groups. As the white blood cell count in this patient group was high (median 42×10^9/L) and *FLT3* (fms-related tyrosine kinase 3) mutations are frequently found in patients with a high white blood cell count, we also analyzed *FLT3* internal tandem duplication (*ITD*) and tyrosine kinase domain (*TKD*) mutations, but *INDO* expression was not significantly different in patients with the mutations of *FLT3* (mean *INDO* expression: *FLT3-ITD* positive patients 11.5, *FLT3-ITD* negative patients 11.6).

When correlating *INDO* expression levels to clinical outcome, patients with prolonged overall survival had low *INDO* levels (Figure 1C). As the maximum *INDO* level in healthy donors was 1.2 relative to *GUS*, we divided the patients into 2 groups; those having *INDO* greater than 1.2 (n=15) or lower than or equal to 1.2 (n=56). There was no difference in median age and white blood cell counts at diagnosis between these groups. However, patients with *INDO* level > 1.2 had a significantly decreased overall survival as compared to patients with *INDO* level lower than 1.2 [7.4 months *vs.* 21.4 months respectively, p=0.01 (Mann-Whitney U)]. Also, relapse-free survival was significantly shortened in patients with *INDO* level greter than 1.2 as compared to patients with *INDO* level lower than 1.2 (6.1 months *vs.* 24.5 months respectively, p=0.025 (Mann-Whitney U)). Note that other cut-off levels such as 0.5 (median of healthy controls) or 1.5 resulted in comparable significant results. *INDO* expression more or less than 1.2 resulted in strongly diverging Kaplan Meier survival curves for overall and relapse-free survival (Figures 1D and 1E).

In a univariate regression model, *INDO* expression level greater than 1.2 was the strongest predictor for overall survival (p<0.001 odds ratio 3.2), as compared to white blood cell count (p=0.038, odds ratio 1.004), age (p=0.012, odds ratio 1.019) and *FLT3-ITD* mutation (p=0.058, odds ratio 1.8). In a multivariate regression model with the same variables, *INDO* expression level greater than 1.2 remained the strongest independent factor predicting survival (p=0.001 vs. p=0.29 (white blood cell count), p=0.005 (age) and p=0.055 (*FLT3-ITD* mutation).

As the groups of patients with good and poor cytogenetic risk profile were too small to use in a multiple regression model, the group with an intermediate cytogenetic risk profile was analyzed (n=40). Also in this group *INDO* expression was a strong predictor for sur-

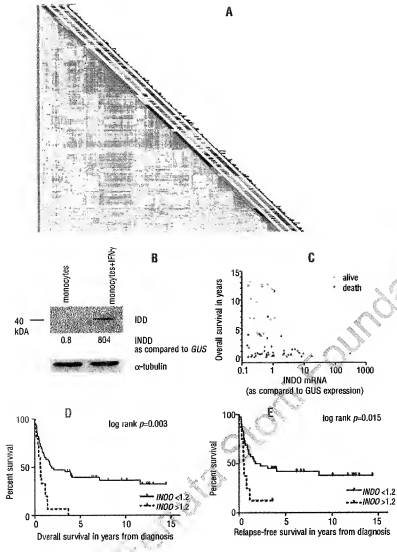

Figure 1. (A) *INDO* expression by microarray and by qPCR correlated to clinical outcome. Unsupervised cluster analyses on the basis of gene-expression profiles of 285 blasts of patients with acute myeloid leukemia. The columns adjacent to the heatmap indicate sample characteristics (column 1: the 16 gene-expression cluster (distinct colors 1-16); column 2: FAB (M0-red, M1-green, M2-purple, M3-orange, M4-yellow, M5-blue, M6-grey); column 3: karyotype (normal-green, inv(16)-yellow, t(8;21)-purple, t(15;17)-orange, 11q23 abnormalities-blue, 7(q) abnormalities-red, +8-pink, complex-black, other-grey)); column 4: *INDO* mRNA expression (probe set ID 210029_at, size of histogram proportional to level of expression). *INDO* expression does not correlate to one of the 16 previously described gene-expression subgroups,[14] the FAB classification or karyotype of the acute myeloid leukemia cases. (B) *INDO* mRNA expression levels (relative to *GUS*) by qPCR nicely correlated to protein levels of indoleamine 2,3-dioxygenase (IDO) as determined by Western Blot on human peripheral blood interferon-γ (IFN-γ) stimulated monocytes. (C) *INDO* expression analyzed by qPCR in 71 patients with acute myeloid leukemia. Patients with prolonged overall survival have low *INDO* expression level. D. and E. *INDO* expression levels analyzed by qPCR; *INDO* expression >1.2 (relative to *GUS*) correlates to significantly shortened overall and relapse-free survival (x- axis is log transformed).

vival (p=0.009, odds ratio 1.146). Myeloid leukemic blasts do express an active form of indoleamine 2,3-dioxygenase protein as previously demonstrated in human myeloid leukemic samples and *in vivo* in mice.[11,12] Moreover, Curti *et al.* demonstrated good correlation between *INDO* mRNA level, indoleamine 2,3-dioxygenase protein expression levels and function in myeloid leukemic samples.

Our findings show that all patients with prolonged overall survival had low *INDO* expression levels and that high *INDO* expression levels were a strong negative predicting variable for worse outcome of patients with acute myeloid leukemia. In a multivariate regression model, a high *INDO* expression level was a stronger predicting variable than white blood cell count, age and *FLT3-ITD* mutational status.

High *INDO* expression was also significantly correlated to a lower complete remission rate (53% for patients with *INDO* expression level greater than 1.2 (p=0.01 (Mann-Whitney U) versus 84% for patients with *INDO* expression level lower than 1.2, p=0.01 (Mann-Whitney U)). This was surprising as it is not expected that the amount of indoleamine 2,3-dioxygenase in blasts is affecting drug sensitivity. In this study, we cannot exclude that high *INDO* expression is a part of a more complex phenotype associated with chemoresistance. However, when only analyzing patients that achieved complete remission, high *INDO* expression was correlated to shortened relapse-free survival.

In the treatment of patients with acute myeloid leukemia, there is a need for additional therapies to prevent relapse in patients that have achieved complete remission. The success of allogeneic stem cell transplantation has demonstrated clearly that immune surveillance plays a role by eradicating minimal residual leukemic cells resulting in prolonged complete remission rates for patients with acute myeloid leukemia. However, allogeneic stem cell transplantation is not available for many patients. For these patients, rehabilitation of autologous immune surveillance, for example

by inhibition of tumor induced indoleamine 2,3-dioxygenase expression could offer new treatment possibilities. As inhibition of indoleamine 2,3-dioxygenase by orally available inhibitors like 1-methyl-tryptophan is effective in mice and synergistic with chemotherapy,[17,18] we now provide further reason for rapid exploration of the introduction of indoleamine 2,3-dioxygenase inhibition in the treatment of patients with acute myeloid leukemia. Besides these opportunities for new immune modulating therapies, measuring *INDO* levels provides useful prognostic information, as a high *INDO* expression level is correlated to a lower complete remission rate, and shorter overall and relapse-free survival of patients with acute myeloid leukemia.

Authorship and Disclosures

MC performed experiments, analyzed results and wrote the paper; AvdL designed research; CH collected samples; JJ performed experiments and analyzed results; AZ performed experiments; RD and PV performed experiments and analyzed results; BL and GO designed research. The authors reported no potential conflicts of interest.

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